



저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:



저작자표시. 귀하는 원저작자를 표시하여야 합니다.



비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.



변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 [이용허락규약\(Legal Code\)](#)을 이해하기 쉽게 요약한 것입니다.

[Disclaimer](#)

獸醫學博士 學位論文

Immune Responses to Stimulation of
***Brucella abortus* Antigens**

*Brucella abortus*의 항원 자극에 의한 면역반응

2019년 8월

서울대학교 대학원

수의과대학 수의병인생물학 및 예방수의학 전공

임 영 빈

Immune Responses to Stimulation of *Brucella abortus* Antigens

A Dissertation

**Submitted to the Faculty of Graduate School of Seoul National
University in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Veterinary Pathobiology and Preventive
Medicine**

**Department of Veterinary Medicine
(Major: Veterinary Pathobiology and Preventive Medicine)
The Graduate School
Seoul National University**

By

Young Bin Im

2019

Abstract

Immune Responses to Stimulation of *Brucella abortus* Antigens

Young Bin Im

(Supervisor: Prof. Han Sang Yoo, D.V.M., Ph.D)

Department of Veterinary Medicine

The Graduate School

Seoul National University

Brucellosis caused by *Brucella* spp. and *Brucella abortus* has been known as a causative organism of zoonotic disease. *B. abortus* causes undulant fever, endocarditis, arthritis and osteomyelitis in man and abortion and infertility in cattle. It easily infects not only animals by ingestion or inhalation bacterium in reproductive tissues, fetal fluids, and mammary gland, but also a man by direct or indirect contact with abortion related discharges and ingestion of unpasteurized dairy products from

the infected animals.

B. abortus which invaded into the hosts through various pathways constructs autologous protection systems to avoid host cell immune system. In addition, the bacterium has been using various factors such as *Brucella*-containing vacuole (BCV), Type IV secretion system (T4SS), Cyclic β -1,2-glucan (c β G), *Brucella*-lipopolysaccharide (Br-LPS) to induce survival/replication of the organism in the host cells. Moreover, it interferes with the signaling pathways of phagocytic cells by regulation of the expression of cytokines in the immune cells of the host cells. A number of studies have been carried out for the prevention and eradication of brucellosis that allows such stealthy invasion and survival/replication. In particular, the mechanism of invasion into host cells and the analysis of immunological characteristics are actively investigated.

Currently, efforts to detect early infection of *B. abortus* have been made. Cellular proteins of *B. abortus* have been received attention as diagnostic antigens through the studies. Based on current knowledge, cellular proteins as recombinant form were used to compare the expression of various genes, especially genes related with cytokines by stimulation of immune cells derived from different host. Also, this study attempted to generate basic information on the development of new diagnostic method(s) and vaccine candidate in *B. abortus* infection.

First, the expressions of IL-1 β , IL-4, IL-6, IL-12p40, IFN- γ , TNF- α and iNOS were analyzed in bovine peripheral blood mononuclear cells. Also, the expression of Bax, Bcl-2 and TLR4 was analyzed after stimulation with *B. abortus* antigens (rMdh,

rOMP28, rRocF, rTsf and r0628). Each *B. abortus* antigens induced different patterns of cytokine expression depending on stimulation time and doses of antigens. Although IL-1 β , IL-4, TNF- α and iNOS gene expressions were not observed, the expressions of IL-6, IL-12p40 and IFN- γ were induced in all stimulated *B. abortus* antigens ($P < 0.05$). The expression of apoptotic genes was not changed except TLR4 ($P < 0.05$).

Second, RAW 264.7 cells, a mouse macrophage cell-line, and mice were stimulated with *B. abortus* antigens (rMdh, rOMP10, rOMP19, rOMP28, rRocF and rTsf) to confirm the difference of immune responses in *in vitro* and *in vivo*. Changes in cytokine production were examined by stimulating with the cellular proteins in both RAW 264.7 cells and naive splenocytes. The immune response was analyzed by ELISA and ELISpot after immunization of mice with the *B. abortus* antigens (rMdh, rOMP10, rOMP19, rOMP28, rRocF and rTsf). The production of cytokines such as TNF- α and IL-6 was increased in stimulated RAW 264.7 cells ($P < 0.05$) while Th1-related cytokines, IFN- γ and IL-2, were induced in naive splenocytes ($P < 0.01$). In addition, the immune responses induced by the *B. abortus* antigens were compared by analyzing the *in vivo* immune response through the difference of IgG secretory cells. As a result, IgG production on the 28th day was significantly increased by immunization with those antigens ($P < 0.05$).

Last, it was studied that the difference in immune responses generated by stimulated with *B. abortus* antigens (rMdh, rOMP10, rOMP19, rRocF and rTbpA) in THP-1 cells, human-derived leukemic monocytes. In particular, it was

investigated to focus on the expression of major immune-regulating factors that play important roles in innate immunity, such as cytokines and Toll-like receptors (TLRs), and acquired immunity in the host. After stimulation of THP-1 cells with *B. abortus* antigens, intracellular cytokine production and TLR expression were increased at different time points (12 and 24 h), and the differences were analyzed by quantified using ELISA and real-time RT-PCR, respectively ($P < 0.05$). In the expression of cytokines, it was observed that the production levels of TNF- α and IL-6 were induced at high levels in THP-1 cells stimulated with *B. abortus* antigens ($P < 0.05$). In addition, expression of TLR8 was significantly increased at 12 h after stimulation with rOMP19 and rMdh ($P < 0.05$). These results suggest the need for further studies on how the two *B. abortus* antigens, rOMP19 and rMdh, are involved in the expression of TLR8 within THP-1 cells.

The results of these studies with immune cells derived from different hosts using the *B. abortus* antigens (rMdh, rTsf, rRocF, r0628, rTbpA, rOMP10, rOMP19 and rOMP28) showed that many of *B. abortus* cellular antigens showed higher antigenicity in different hosts. It indicated that rMdh and rOMP19 of these *B. abortus* antigens exhibits higher immunogenicity than other antigens. In particular, rMdh showed the highest immunogenicity to all immune cells used in this study. This study demonstrated the possibility of *B. abortus* antigen as an intracellular immune response modulator as well as a candidate for high immunogenicity antigens. Therefore, these findings would be useful for understanding basic immune responses, development of diagnostic method and vaccine candidate in *B. abortus* infection in

the future.

Keywords: Brucellosis, *Brucella*, *B. abortus* antigens, Immune responses, Th1, Th2, Cytokines, Immune cells.

Student Number: 2010-31124

Contents

Abstract	i
Contents	vi
List of Figures	ix
List of Tables	xii
List of Abbreviations	xiii
 General Introduction	 1
Literature Review	4
I. <i>BRUCELLA</i> PATHOGENESIS.....	4
1.1. Origin of <i>Brucella</i>	4
1.2. Pathogenic Mechanism of <i>Brucella</i>	6
II. <i>BRUCELLA</i> IMMUNE RESPONSES.....	14
2.1. Innate Immune Response.....	15
2.2. Adaptive Immune Response	18
III. CURRENT IMMUNOLOGICAL STUDIES OF <i>BRUCELLA</i>	22
 Chapter I. Expression of Cytokine and Apoptosis-related Genes in Bovine Peripheral Blood Mononuclear Cells Stimulated with <i>Brucella abortus</i> Antigens	
Abstract.....	26
1.1. Introduction	28
1.2. Materials and Methods	30
1.3. Results	34

1.4. Discussion	37
 Chapter II. Th2-related Immune Responses in <i>in vitro</i> and <i>in vivo</i> Stimulated with <i>Brucella abortus</i> Antigens, Malate dehydrogenase (Mdh), Elongation factor (Tsf) and Arginase (RocF)	
Abstract	54
2.1. Introduction	56
2.2. Materials and Methods	58
2.3. Results	64
2.4. Discussion	67
 Chapter III. Comparative Analysis of Immune Responses in <i>in vitro</i> and <i>in vivo</i> after stimulation of <i>Brucella abortus</i> Outer Membrane Antigens, OMP10, OMP19 and OMP28	
Abstract	77
3.1. Introduction	79
3.2. Materials and Methods	80
3.3. Results	86
3.4. Discussion	89
 Chapter IV. Cytokines Production and Expression of Toll-like Receptors in Human Leukemic Monocyte Cells, THP-1, Stimulated with <i>Brucella abortus</i> Antigens	
Abstract	100
4.1. Introduction	102
4.2. Materials and Methods	104

4.3. Results	108
4.4. Discussion	111
General Conclusion	120
References	124
국문초록	152

List of Figures

- Figure 1. Schematic representation of *Brucella* cell envelope and molecules required for controlling its intracellular trafficking
- Figure 2. Working model of *Brucella* intracellular trafficking in macrophage cells
- Figure 3. *Brucella* invasion and intracellular trafficking in host mammalian cells
- Figure 4. The key mechanisms in different cells of the innate and adaptive immune system after *B. abortus* infection
- Figure 5. Strategies of *Brucella* to evade the innate and adaptive immunity
- Figure 6. Analysis of the purified recombinant proteins
- Figure 7. Gene expression of inducible nitric oxide synthase (iNOS) in bovine PBMC
- Figure 8. Gene expression of interleukin-1 β (IL-1 β) in bovine PBMC
- Figure 9. Gene expression of interleukin-4 (IL-4) in bovine PBMC
- Figure 10. Gene expression of interleukin-6 (IL-6) in bovine PBMC
- Figure 11. Gene expression of interleukin-12p40 (IL-12p40) in bovine PBMC
- Figure 12. Gene expression of interferon- γ (IFN- γ) in bovine PBMC
- Figure 13. Gene expression of tumor necrosis factor- α (TNF- α) in bovine PBMC
- Figure 14. Gene expression of Bax in bovine PBMC
- Figure 15. Gene expression of Bcl-2 in bovine PBMC
- Figure 16. Gene expression of Toll-like receptor 4 (TLR4) in bovine PBMC

- Figure 17. Analysis of purified recombinant Mdh, Tsf, and RocF of *Brucella abortus*
- Figure 18. Productions of TNF- α in RAW 264.7 cells stimulated with recombinant proteins
- Figure 19. Productions of IFN- γ and IL-2 by naïve mouse splenocytes
- Figure 20. Numbers of antigen-secreting cells (ASCs) in the splenocytes of mice immunized with rMdh, rTsf, or rRocF of *Brucella abortus*
- Figure 21. Humoral immune responses (IgM, IgG, IgG2a and IgG1) in mice immunized with rMdh, rTsf, or rRocF
- Figure 22. Analysis of purified recombinant OMP10, OMP19, and OMP28 of *Brucella abortus*
- Figure 23. Production of nitric oxide (NO) and cytokines in RAW 264.7 cell after stimulation with recombinant proteins
- Figure 24. Production of IFN- γ and IL-2 by naïve mice splenocytes
- Figure 25. Detection of antigen-specific antibodies in immunized mice after reaction with recombinant TF protein
- Figure 26. Humoral immune responses induced in mice by immunization with rOMP10, rOMP19, and rOMP28 of *Brucella abortus* (IgM and IgG)
- Figure 27. Humoral immune response induced IgG2a and IgG1
- Figure 28. Analysis of *Brucella abortus* recombinant cellular proteins by SDS-PAGE (A) and Western blot (B)

Figure 29. The production of cytokines in THP-1 cells (A) TNF- α , (B) IL-1 β , (C) IFN- γ and (D) IL-6, after stimulation with *B. abortus* recombinant proteins

Figure 30. The expression of (A) TLR3, (B) TLR4, (C) TLR7, (D) TLR8, (E) MyD88, and (F) IRF4 in THP-1 cells after stimulation with *B. abortus* recombinant proteins

List of Tables

- Table 1. List of different *Brucella* species and their natural hosts
- Table 2. Primer sequences for cloning *Brucella abortus* genes
- Table 3. The primer sets for real-time PCR
- Table 4. Primer sequences used for the cloning *Brucella abortus* genes
- Table 5. Number of antigen-secreting cells (ASC) from splenocytes of mice immunized with rOMP10, rOMP19, and rOMP28 of *Brucella abortus* at 28 days post immunization
- Table 6. Nucleotide sequence of primers used in this study

List of Abbreviations

ASC	antigen-secreting cell
Bax	BCL2 associated X
BCV	<i>Brucella</i> -containing vacuole
Bcl-2	B-cell lymphoma 2
Br-LPS	<i>Brucella</i> -lipopolysaccharide
BvrR	<i>Brucella</i> virulence-related regulatory
BvrS	<i>Brucella</i> virulence-related sensory
cβG	Cyclic β -1,2-glucan
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTPase	GTP-binding proteins
ICSBP	Interferon consensus sequence binding protein
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1

IgG2a	Immunoglobulin G2a
IgM	Immunoglobulin M
IL-1β	Interleukin-1beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-12p40	Interleukin-12p40
iNOS	Inducible nitric oxide synthase
IRF	IFN regulatory factor
LAMP	Lysosomal-associated membrane protein
LPS	Lipopolysaccharide
Mdh	Malate dehydrogenase
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response 88
NO	Nitric oxide
NK	Natural killer
OMPs	Outer membrane proteins
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid

RNI s	Reactive nitrogen intermediates
RocF	Arginase
ROI s	Reactive oxygen intermediates
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Tc1	T lymphocytes 1
T4SS	Type IV secretion system
Th	Helper T cells
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
Tsf	Elongation factor Ts

General Introduction

Brucellosis caused by *Brucella*, which is Gram-negative and has two chromosomes, is well known as a common zoonosis. Twelves of *Brucella* species have been identified since the first known brucellosis in 1908 as Malta fever; *Brucella melitensis* (sheep and goats), *B. suis* (hogs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dog), *B. neotomae* (wood rats), *B. ceti* (seals), *B. pinnipedialis* (pinnipeds), *B. inopinata* (Breast implant infection), *B. microti* (common vole), *B. vulpis* (red fox), and *B. papionis* (baboon) [Foster *et al.*, 2007; Ko *et al.*, 2003, Scholz *et al.*, 2010; Whatmore *et al.*, 2014; Scholz *et al.*, 2008; Scholz *et al.*, 2016]. It is also known that *Brucella* penetrates into cells and is capable of proliferation. Abortion and infertility are known to be typical clinical symptoms of brucellosis in livestock, and osteoarticular (ie, peripheral arthritis, sacroiliitis, spondylitis) is known to occur in human infection [Baladi *et al.*, 2013].

The immune response at the cellular level in *Brucella* infections is the earliest defense system. In particular, studies on the intracellular immune response using *B. abortus* have been performed recently, and studies on the changes of immune responses from the early stage of infection to the time course have been actively conducted [Mol *et al.*, 2014; Hielpos *et al.*, 2018]. Furthermore, various studies have been reported on various changes in intracellular invasion of *Brucella* [Ferrero *et al.*, 2012; Scian *et al.*, 2012; Li *et al.*, 2015; Reyes *et al.*, 2017]. Several studies have

been reported on the differences in the amount of cytokine expression by immune cell stimulation using *Brucella* recombinant protein, the difference in expression protein in the microarray, and the immunological induction ability according to changes in immunoglobulin [Tan *et al.*, 2012; Tian *et al.*, 2013; Im *et al.*, 2016a; Im *et al.*, 2016b; Im *et al.*, 2017; Im *et al.*, 2018a; Im *et al.*, 2018b; Shim *et al.*, 2018; Soh *et al.*, 2019]. In addition, in the experiment using the *Brucella* mutant, the immune responses in the cells through the control and mutation stimuli were analyzed to show differences in specific protein changes in the cells of *Brucella* [Tian *et al.*, 2014; Park *et al.*, 2018; Jung *et al.*, 2018; Li *et al.*, 2018].

In the case of brucellosis, an intracellular bacterium, immune responses of host cells can be avoided after penetration into cells. In other words, this stealthy ability of *Brucella* is able to make possible to penetrate and replicate within the cell. In *Brucella*, *Brucella*-containing vacuole (BCV), Type IV secretion system (T4SS), Cyclic β -1,2-glucan (c β G), and *Brucella* lipopolysaccharide (Br-LPS) play a major role to avoid immune mechanism within the host. Some studies have been reported on the intracellular changes of *Brucella* host invasion using mice [Alva-Pérez *et al.*, 2014; Vitry *et al.*, 2014; Reyes *et al.*, 2017]. In particular, infection with *Brucella* is identified in three steps in a mouse. The first step is the step of invasive the host by the bacteria, which usually occurs primarily within two days of infection. The second stage is the period of bacterial invasion into host cells, replication in the reticuloendothelium and reproductive organs, which occurs mainly between two weeks and three weeks, in the acute phase. The last step is a chronic stage with

duration of up to six months to a year, with long-term pathologic symptoms in organs [Plommet *et al.*, 1988; Martirosyan *et al.*, 2011; Grillo *et al.*, 2012; Martirosyan and Gorvel, 2013]. Through the stealthy survival and replication ability of *Brucella*, it is possible for *Brucella* to stay in the host cells for long periods of time. Thus, studies have been actively conducted to analyze the roles of certain *Brucella* elements and to control them to treatment of brucellosis and to find vaccine candidates [Vishnu *et al.*, 2015; Truong *et al.*, 2016; Kim *et al.*, 2017].

Many studies have been conducted to analyze the intracellular mechanism and immune response of brucellosis. This study tried to analyze the immune response from various immune cells after stimulation of *Brucella* recombinant proteins. First, bovine peripheral blood mononuclear cells (bPBMCs) were stimulated with recombinant proteins and the expression of cytokines and genes in the cells was analyzed by real-time PCR. Second and third, the immune response in mouse macrophage cells (RAW 264.7 cells) stimulated with *Brucella* cellular proteins and the immune response in RAW 264.7 cells after stimulation with *Brucella* outer membrane proteins were analyzed, respectively. Finally, the immune responses of human leukemic monocyte cells (THP-1 cells) were analyzed after stimulation of the *Brucella* recombinant proteins.

Therefore, the aim of this study was to investigate immune response regulating activity of *Brucella* cellular antigens and its potential as diagnosis and/or vaccine candidate using the *Brucella* cellular antigens through these studies.

Literature Review

I. *BRUCELLA* PATHOGENESIS

1.1. Origin of *Brucella*

Brucellosis, which is still occurring in many parts of the world, is known to be caused by *Brucella* as a common infectious disease. This persistent brucellosis causes economic problems such as reduced breeding and production in animals. At the beginning of 1960, only three species of *Brucella*, *B. abortus*, *B. melitensis*, and *B. suis*, were classified. Later, *Brucella* species were categorized more through continuous *Brucella* studies and up to now, twelve *Brucella* species have been categorized from different hosts (Table 1) [El-sayed and Awad, 2018].

In general, for humans, the infection of *Brucella* occurs through the consumption of unsterilized dairy products produced from livestock infected with brucellosis, or through direct contact with tissues or bodily fluids of infected animals. It can also be transmitted through the surface of the mucosa and infected by carelessly being stabbed by the needles of the brucellosis vaccine. Infections caused by breast milk or sexually transmitted diseases between humans and humans are rare [Celebi *et al.*, 2007; Meltzer *et al.*, 2010].

Table 1. List of different *Brucella* species and their natural hosts

<i>Brucella</i> species	Colony type	Natural host	Zoonoses	Year of first isolation
<i>B. melitensis</i> (bv1-3)	Smooth	Goat and sheep	+++	Bruce (1893)
<i>B. abortus</i> (bv 1–6, 7, 9)	Smooth	Cattle	++	Schmidt (1901)
<i>B. suis</i> biovar				Huddleson (1929)
<i>B. suis</i> (bv 1–3)	Smooth	Pig	++	
<i>B. suis</i> (bv 2)	Smooth	Wild boar, Hare	+	
<i>B. suis</i> (bv 4)	Smooth	Reindeer, Caribou	++	
<i>B. suis</i> (bv 5)	Smooth	Rodent	–	
<i>B. ovis</i>	Rough	Sheep	–	Buddle (1956)
<i>B. neotomae</i>	Smooth	Desert rat	+	Stoenner and Lackman (1957)
<i>B. canis</i>	Rough	Dog	+	Carmichael and Bruner (1968)
<i>B. ceti</i> (<i>B. delphini</i>)	Smooth	Dolphins	+	Foster <i>et al.</i> (2007)
<i>B. pinnipedialis</i> (<i>B. phocae</i>)	Smooth	Seals	+	Foster <i>et al.</i> (2007)
<i>B. microti</i>	Smooth	Wild voles	(?)	Scholz <i>et al.</i> (2008)
<i>B. inopinata</i>	Smooth	Human	++	Scholz <i>et al.</i> (2008)
<i>B. papionis</i>	(?)	Baboons (<i>Papio</i> spp.)	(?)	Whatmore <i>et al.</i> (2014)
<i>B. vulpis</i>	(?)	Red foxes (<i>Vulpes vulpes</i>)	(?)	Scholz <i>et al.</i> (2016)
N.N.	Smooth	Frog	(?)	Soler-Lloréns <i>et al.</i> (2016)

(Adapted from El-sayed and Awad, 2018).

1.2. Pathogenic Mechanism of *Brucella*

In general, clinical infections of *Brucella* occur through mucosa and tissue along with various bacteria. Martirosyan *et al.* has summarized the process of *Brucella* infection as follows. First, *Brucella* has to be strong and protect his being. Second, *Brucella* should adapt to host cells without causing a distraction and release host damage materials while avoiding colliding with self-protection. Finally, *Brucella* has to give up, convert, or hide molecular patterns related to pathogens, except that they conflict with the first or second law [Martirosyan *et al.*, 2011]. Thus, *Brucella* can survive and replicate within the host through this process.

Brucella is involved in many factors to stably parasitize the host cell while protecting itself in the invasion process. In particular, *Brucella* has excellent resistance ability to many bactericidal substances generated to counter external bacterial infection within the host [Martinez de Tejada *et al.*, 1995; Freer *et al.*, 1996; Paramo *et al.*, 1998; Manterola *et al.*, 2007; Ettema *et al.*, 2009]. This property is related to the structure of cell membranes with high hydrophobicity. Specifically, *Brucella*'s outer membrane is composed of phospholipids, ornithine lipids, lipoproteins, and non-canonical lipopolysaccharide (LPS) molecules. Lipid A and core oligosaccharides of *Brucella* LPS (Br-LPS) reduce the number of negatively charged sugars [Conde-Álvarez *et al.*, 2013]. These features help to reduce negative charge on smooth bacterial surfaces overall [Conde-Álvarez *et al.*, 2013]. As a result, *Brucella* does not bind complement, microbicidal defensins, bactericins,

cathelicidins, or any other cationic bactericidal molecule. This particular cell envelope structure makes *Brucella* highly resistant to all highly active molecules for pathogenic bacteria such as complement lysozyme, phospholipases, and lactoferrin, as well as most germicidal materials containing lysosomal extracts (Figure 1). As a result of separating Br-LPS having this role from *Brucella*, isolated Br-LPS almost did not bind complement [Barquero-Calvo *et al.*, 2007; Barquero-Calvo *et al.*, 2009]. In addition, *Brucella* mutants deficient in O-chain with core defects or lipid A imbalance are more susceptible to bactericidal substances and complement.

Brucella should avoid the fusion of the *Brucella*-containing vacuole (BCV) with the host cell lysosomes, thereby protecting and moving to a place where it can replicate itself. To do this, several processes of the cells must be destroyed. For the first few minutes after penetration into the non-professional phagocyte, both the virulent *Brucella* and the attenuated vaccine strain (*B. abortus* 19) also interact with the early endosomal compartments. This was confirmed by expression of the early endosomal markers, transferrin receptor, GTP-binding protein rab5, or early endosomal antigen in early BCVs [Pizarro-Cerdá *et al.*, 1998; Delrue *et al.*, 2001]. In addition, this phenomenon becomes to decrease after 10 minutes of infection. Nevertheless, acidification of the BCV compartment is needed early in the maturation of BCV [Porte *et al.*, 1999] and acquisition and maintenance of LAMP-1 (lysosomal-associated membrane protein-1) are required for 4 h after infection. Simultaneously as LAMP-1 decreases, BCVs acquire an ER marker (glucose 6-

phosphatase) such as calreticulin or calnexin. Through this process, *B. abortus* moves to a safe place (Figure 2) [de Figueiredo *et al.*, 2015].

In the intracellular replication of *B. abortus* in the early stages of infection, small GTPase Sar1 is present as an important protein in the presence of ER membrane and perfusion towards Golgi [Stephens *et al.*, 2000; Barlowe *et al.*, 2002; Celli *et al.*, 2005]. In addition, the ER membrane contains not only ER Chaperone (calnexin, calreticulin, the translocator sec61 β and an ER-resident enzyme protein disulfide isomerase (PDI)) for replication of *B. abortus* [Pizarro-Cerdá *et al.*, 1998; Pizarro-Cerdá *et al.*, 1999; Commerci *et al.*, 2001; Celli *et al.*, 2005], but also two non-ER related cell proteins (GAPDH and GTPase Rab2), which are essential for the survival of *B. abortus* (Figure 3) [Fugier *et al.*, 2009].

Several factors are involved in intracellular trafficking and survival of *Brucella* [Gomez *et al.*, 2013]. However, the exact control mechanism for these is still unknown. One well-known element is the Type-4-secretion system (T4SS), which is encrypted in the VirB region [O'Callaghan *et al.*, 1999; Celli *et al.*, 2003]. This region consists of 12 genes that form the operon [Porte *et al.*, 1999]. Although VirB-deficient mutants have the same cell penetration rate as wild *Brucella* [Celli *et al.*, 2003; Fontes *et al.*, 2005], it does not promote BCV and ER fusion then fuses with the lysosome [Arellano-Reynoso *et al.*, 2005]. Nonetheless, when infected the wild *Brucella* and the mutant at the same time, the mutant can move to the replication

niche. This suggests that the VirB system is necessary for trafficking but is not an essential factor for late replication.

Cyclic β -1,2-glucan (c β G) acts as an osmotic pressure regulator as another factor controlling *Brucella* trafficking [Arellano-Reynoso *et al.*, 2005]. c β G has a structure similar to β -cyclodextrin. β -cyclodextrin shows a high affinity with cholesterol present in the cell membrane and allows cell lysis through the destruction of the lipid structure. On the other hand, c β G exhibits a low affinity for cholesterol. And it can migrate lipid structure, but do not have any toxicity or dissolution in cells. In addition, c β G is not transported by the VirB system, and this polysaccharide is not present in the outer membrane fraction shed by *Brucella* [Aragón *et al.*, 1996]. One of the important systems involved in the intracellular survival of *Brucella* is *Brucella* virulence-related regulatory (BvrR) and sensory (BvrS), two component regulation systems very similar to the putative system present in the animal pathogen *Bartonella henselae* [Quebatte *et al.*, 2010]. Both BvrR and BvrS mutants are less invasive than wild-type strains and both mutants are not replicated in phagocytes or non-phagocytes and are targeted to lysosomes for degradation [Sola-Landa *et al.*, 1998]. Moreover, the BvrR/BvrS mutant cannot directly activate the Cdc42 small GTPase upon cell contact and remains bound to the host cell surface without the ability to penetrate the cell. These events indicate that these two component systems regulate the expression of molecular determinants required for invasion [Guzmán-Verri *et al.*, 2001]. It has also been shown that the BvrR regulatory protein directly

interacts with and regulates the promoter region of the VirB operon, and the expression of the transcriptional activator VjbR, which affects the VirB operon, is mediated by the regulatory action of the BvrS/BvrR system [Delrue *et al.*, 2005]. This property demonstrates that BvrS/BvrS controls the expression of several systems essential for intracellular migration through direct and indirect mechanisms. Besides this, *bacA* gene [LeVier *et al.*, 2000], *cydB* gene [Endly *et al.*, 2001], *purE* gene [Crawford *et al.*, 1996], *aroC* gene [Foulongne *et al.*, 2001], Heat shock proteins and Cu-Zn superoxide dismutase (SOD) [Teixeira-Gomes *et al.*, 2000] have been known factors to influence the pathogenesis of *Brucella*.

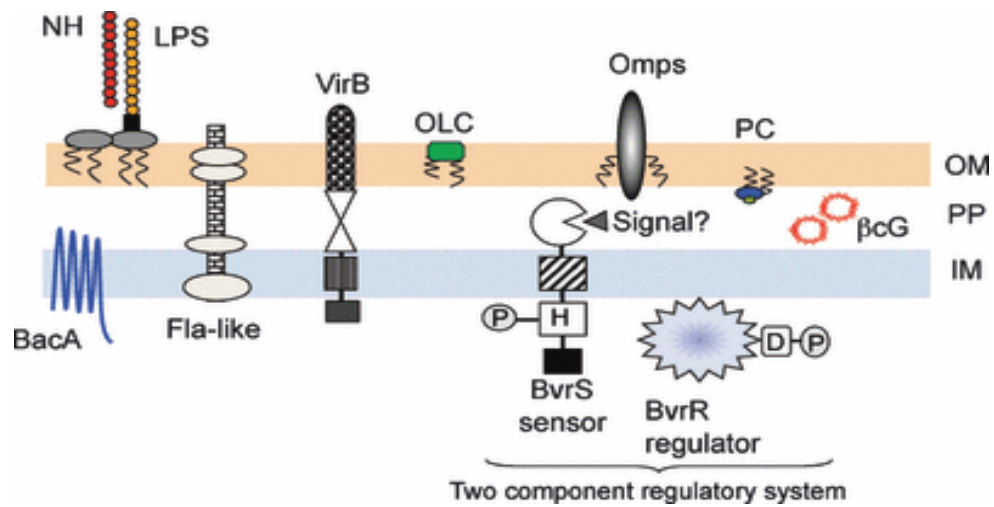


Figure 1. Schematic representation of *Brucella* cell envelopes and molecules required for controlling its intracellular trafficking. (Adapted from Martirosyan *et al.*, 2011).

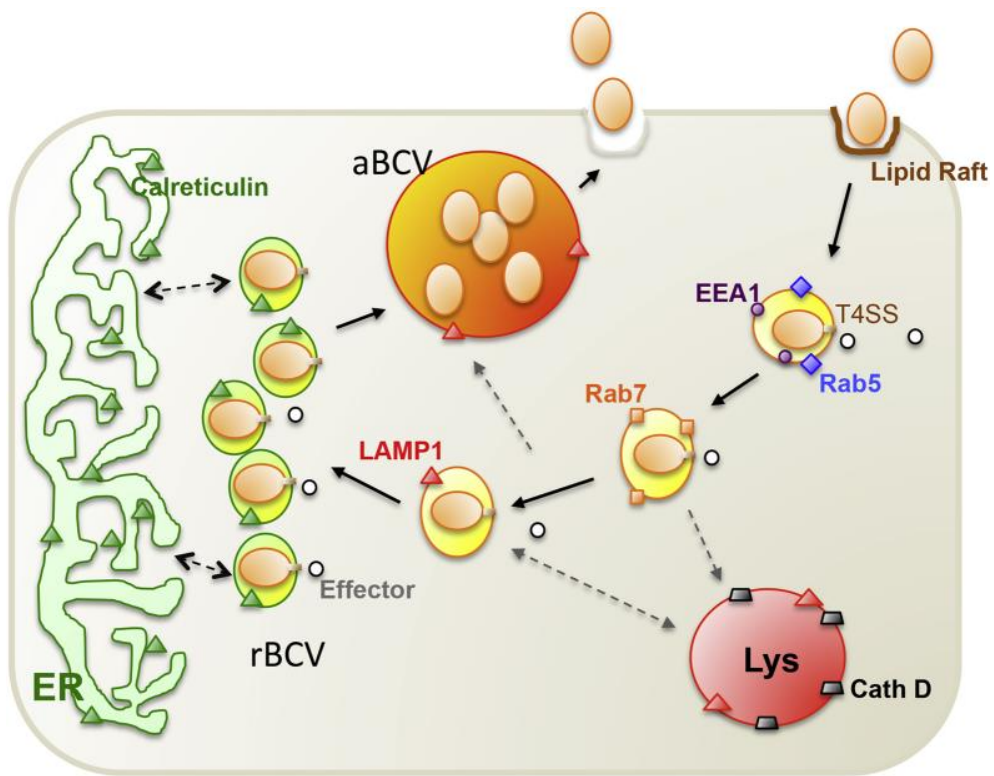


Figure 2. Working model of *Brucella* intracellular trafficking in macrophage cells.

(Adapted from de Figueiredo *et al.*, 2015).

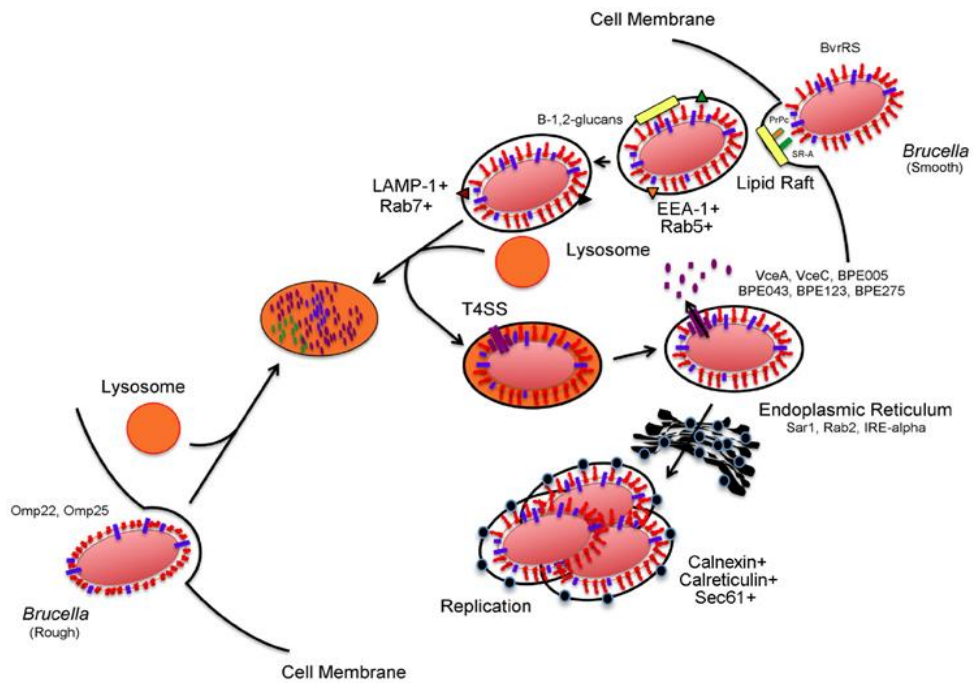


Figure 3. *Brucella* invasion and intracellular trafficking in host mammalian cells.

(Adapted from Gomez *et al.*, 2013).

II. *BRUCELLA* IMMUNE RESPONSES

Brucella has the ability to evade immune responses in cells by developing a well-organized strategy that can interfere with the innate immune recognition that ultimately prefers the environment to evolve and adaptive immune responses to host cells after infection [Diacovich and Gorvel, 2010]. In particular, antibodies of the IgG, IgM or IgA class play a role in defending the host from brucellosis. Anti-*Brucella* antibodies are proteins that cause aggregation, complement fixation, and precipitation when they react with homologous antigens from *Brucella*. Most reactive antibodies were induced by *Brucella* lipopolysaccharide (Br-LPS) rather than cytoplasmic protein. The IgM antibody to Br-LPS appears for the first time after infection and gradually increases in the course of acute infection whereas the IgG antibody appears later in the *Brucella* infection and is caused by the *Brucella* cytoplasmic protein. Therefore, based on this, most of the sera can be used to distinguish host infections based on LPS. Cytoplasmic proteins do not have a single antigenic determinant that distinguishes between infected human and naturally infected animals. Therefore, the identification of important antigenic determinants among strains of *Brucella* spp. or the discovery of

suitable substances that do not cross-react with other bacteria is important for the diagnosis of brucellosis, and such studies have been still undergoing.

2.1. Innate Immune Response

The first lines of defense against *Brucella* are the phagocytosis by cells (neutrophils, macrophages, dendritic cells (DC), natural killer (NK) cells), secretion by cytokines and chemokines, and by the typical molecular recognition of pattern recognition receptors (PRRs) in microbes, pathogen-associated molecular patterns (PAMPs) and complement systems [Diacovich and Gorvel, 2010]. Neutrophils are one of the most important phagocytic cells in the innate immune response to microbial pathogens. This is the first immune-related cell encountered in host cell infection of *Brucella*. In brucellosis, however, these cells are not stimulated for effective degranulation. For example, *B. abortus* inhibits the myeloperoxidase H₂O₂-halide antimicrobial system in bovine neutrophils by releasing GMP and adenine and inhibiting degranulation [Canning *et al.*, 1985]. Although this pathogen is not replicated in neutrophil cells, it can survive in the early stages of infection and resist death [Barquero-Calvo *et al.*, 2007].

In addition to neutrophils, there are activated NK cells that act as the first line of defense against *Brucella* and killing infected targets [Fernandes *et al.*, 1995]. *Brucella* activates NK cells by inducing interleukin-2 (IL-2)-releasing antigen-

presenting cells and it converts NK cells into killer cells by IL-2 activation, IFN- γ secretion, and production, which play an important role in Th1 or Tc1 expression [Gao *et al.*, 2011]. In human NK cells, the cytotoxicity was increased when NK cells were treated with interleukin-12 (IL-12) obtained from a macrophage stimulated with heat killed *B. abortus in vitro*. It suggests that NK cells are a more potent role in human brucellosis [Zaitseva *et al.*, 1996]. On the other hand, the role of NK cells in mouse brucellosis is not much critical [Fernandes *et al.*, 1995].

Macrophages and dendritic cells are considered to be a key component of the innate immune response to intracellular bacteria such as *Brucella*. Within hours of invading into host cells, a few pathogens reach replication available niche during 80-90% of pathogens are killed by macrophages and dendritic cells [Watarai *et al.*, 2002]. In a bactericide function in macrophage, reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) induced by gamma interferon (IFN- γ) and tumor necrosis factor (TNF- α) play an important role [Jiang *et al.*, 1993]. In addition, for Interferon (IFN) signaling, the second transcription factor, IFN regulatory factors (IRFs) mediate various phenotypes induced by IFN. Expression of IRF-1, IRF-2, and ICSBP among IRFs is induced by IFN- γ , and IRF-1- and ICSBP-deficient mice are susceptible to *B. abortus* when ROI and RNI were absented [Ko *et al.*, 2002].

Lipid A, which plays an important role in immune evasion through TLR4, was produced in *Brucella*. *Brucella* contains a longer fatty acid residue (C₂₈) compared

to other intestinal bacterial LPS (C₁₂-C₁₆). It reduces endotoxicity by reducing TLR4 antagonism due to this alteration of the LPS structure [Lapaque *et al.* 2009]. *B. abortus* mutants lacking lipid A with the C₂₈ acyl chain have greater inflammatory status than wild-type strains. It has also decreased infectivity in BALB/c mice and macrophages [Parent *et al.*, 2007].

Brucella can suppress innate immune signals by producing PAMPs with decreased TLR agonist activity through TIR domain-containing proteins, Btp1 in *B. abortus* and TcpB in *B. melitensis*. However, although the detailed mechanism of this protein has not been fully elucidated, TIR domain-containing protein TcpB is known to inhibit TLR signaling through interaction with adapter proteins TIRAP/Mal and MyD88 in host cells [Snyder *et al.*, 2014].

Br-LPS, a stealth invader, plays a crucial role in reducing the deposition of complement component C3 [Barquero-Calvo *et al.*, 2007]. C3 is covalently bound to the hydroxyl moiety present on the surface of the bacteria. The production of pro-inflammatory complement products such as C3a and C5a is prevented by C3 binding to the *Brucella* O-antigen. Previous study has shown that O-antigens play an important role in promoting non-inflammatory responses through lipid raft microdomains and macrophage class A scavenger receptors SR-A [Kim *et al.*, 2004]. These studies of innate immunology of *Brucella* imply that the key strategy that *Brucella* uses to regulate innate immune mechanisms is the inhibition to TLRs, the complement system and phagocytic cells such as macrophage, DC, and NK cell etc

[Dorneles *et al*, 2015]. Therefore, it is necessary to study the precise mechanism of action of these innate immune responses and the complex immune responses that occur in the *in vivo* system.

2.2. Adaptive Immune Response

Even if innate immunity effectively controls the replication of *Brucella* in the acute phase of infection, an adaptive immune response is an essential mechanism in the chronic phase [Baldwin and Goenka, 2006]. Moreover, adaptive immune responses are important for providing memory function, a key element of immunization when pathogens invaded again. *Brucella* developed several strategies to avoid host defense mechanisms and establish chronic infections [Ahmed *et al.*, 2016]. First, IFN- γ produced by CD4⁺, CD8⁺, and T cells activates bactericidal functions in macrophages to interfere with the cellular survival of *Brucella*. Second, infected macrophages were killed due to the cytotoxicity of CD8⁺ and T cells. Finally, Th1 type antibody isotype such as IgG2a and IgG3 promotes phagocytosis by opsonizing of pathogens. In brucellosis, these three mechanisms are well known for adaptive immune responses. In addition, cytokine expression such as IL-12, IFN- γ , and TNF- α induces not only innate and adaptive immune responses between *Brucella* and host cells but also regulates the activity and/or action of immune-related cells [Martirosyan *et al.*, 2011; Durward *et al.*, 2012]. Br-LPS successfully

elevates Th1 type cytokine responses such as IL-10 and IFN- γ [Kianmehr *et al.*, 2015] in *Brucella* infection of macrophages during controlling the expression of IL-12, especially [Fahel *et al.*, 2015]. In *Brucella* infection, cytokines not only control innate and adaptive immune responses but also play an important role in directly among immune-related cells in the host. IL-12 is a major factor produced by B cells and macrophage and is lead in inducing the Th1 response, eventually expressing IFN- γ in host T cells. In addition to IL-12, there are other cytokines involved in adaptive immune responses and it was studying as important through differences in the amount of expression given by different pathogens. [Pizarro-Cerdá *et al.*, 1999; Hielpos *et al.*, 2018].

Some cells such as DCs play an important role in the initiation and regulation of these adaptive immune responses [Kapsenberg, 2003]. To avoid the function of these cells, intracellular pathogens established several mechanisms. In particular, *Brucella* has established several strategies for confirming the transition from the innate immune system to the adaptive immune system for avoidance of the host immune system. Recently, some evidence has confirmed the efficient propagation of this pathogen in DCs *in vitro* [Archambaud *et al.*, 2010] and *in vivo* [Salcedo *et al.*, 2008]. It indicates that *Brucella* can prevent the development of a protective Th1 immune response by avoiding the induction of IL-12 and T cell stimulating the activity of infected DCs [Salcedo *et al.*, 2008]. In addition, the ability of the *Brucella*-infected DC to mature and express MHC class II, which represents a specific T cell, for an

exogenous protein antigen is decreased [Salcedo *et al.*, 2008; Billard *et al.*, 2007; Gomez *et al.*, 2013]. Therefore, studies of the immune mechanism in *Brucella* infection need to accurately identify of *Brucella* feature when they invade to the host, time-dependent manner. Furthermore, there is a need to continuously study the method of blocking *Brucella* more effectively at the early stage of infection and intracellular survive and replicate stages (Figure 4).

III. CURRENT IMMUNOLOGICAL STUDIES OF *BRUCELLA*

Brucellosis has continuously emerged and it is a zoonosis caused economic loss and social issue. However, the treatment methods that are suitable to the patient, or effective vaccines have not been universalized until now. In contrast, *Brucella* has a remarkable strategy of avoiding host immune responses and promoting the formation of chronic infections. Therefore, host cells have developed complex immune mechanisms to survive against pathogens with this ability.

During intracellular replication, *Brucella* exhibits typical tissue affinities in lymph nodes and reproductive organs, helping to avoid inherent adaptive immune mechanisms in the host to establish clinical disease manifestations and pathogenesis. Symptoms in the early stages of *Brucella* infection observed in humans are fatigue, fever, anorexia, myalgia, and sweating. And the mononuclear phagocytic system, including bone marrow, lymph nodes, liver, and spleen, is continuously observed in the chronic stage. In the early stage of *Brucella* infection, it modulates the host immune response mechanism to rapidly migrate the mucosal immune barrier and form endocytosis by mucosal macrophages and DCs. *Brucella* protects itself by infecting host cells and limiting the role of PRR, including the complement system and TLR signaling pathways. In addition, Br-LPS exhibits non-canonical structural differences that inhibit antigen presentation to T cells and attenuate innate and adaptive immune mechanisms. Specifically, Br-LPS inhibits the activation of DC to

establish a defensive Th1 immune response by preventing T-cell stimulation, which releases IL-12 through destroying the immune response. *Brucella* also regulates MHC-I and MHC-II expression promoted by IFN- γ dependent on cytokine regulation. In addition, *Brucella* has a variety of survival strategies such as inhibiting apoptosis, avoiding immune responses and establishing chronic infections (Figure 5).

Recently, studies on intracellular survival and replication and immune responses of the *Brucella* are steadily investigating. One of a candidate in *Brucella* substances is that *Brucella* recombinant proteins which have been continuously using for developing *Brucella* vaccine and analysis of immune responses within the host. In recent years, many studies have been actively conducted to examine the possibility of identifying novel *Brucella* antigens as candidates for the development of vaccines and effective immune responses in host cells using *Brucella* cellular proteins including outer membrane proteins such as Mdh, TbpA, RocF, OMP10, and OMP19 [Lim *et al.*, 2012b; Simborio *et al.*, 2015; Im *et al.*, 2017; Im *et al.*, 2018]. In addition, it was reported as a possible candidate substance for *Brucella* diagnosis by analyzing changes in immune response or gene expression in different hosts stimulated with *Brucella* recombinant protein or through changes in immune responses by mucosal immunization with *Brucella* recombinant protein nanoparticle. [Im *et al.*, 2016a; Im *et al.*, 2018b; Soh *et al.*, 2019]. In addition, vaccine candidate research using *Brucella* mutants [Bao *et al.*, 2017; Zhang *et al.*, 2017; Li *et al.*, 2017; Li *et al.*, 2018]

and study about small RNA role in *Brucella* infection are active [Eskra *et al.*, 2003; Xu *et al.*, 2018]. Particularly, identifying the role of microRNAs in autophagy and apoptosis in the cellular survival of *Brucella* will be a part of understanding the immune mechanism of *Brucella* infection. Ultimately, the study of immune responses in brucellosis is still the biggest challenge in the field of studying *Brucella*, as well as a task to be solved.

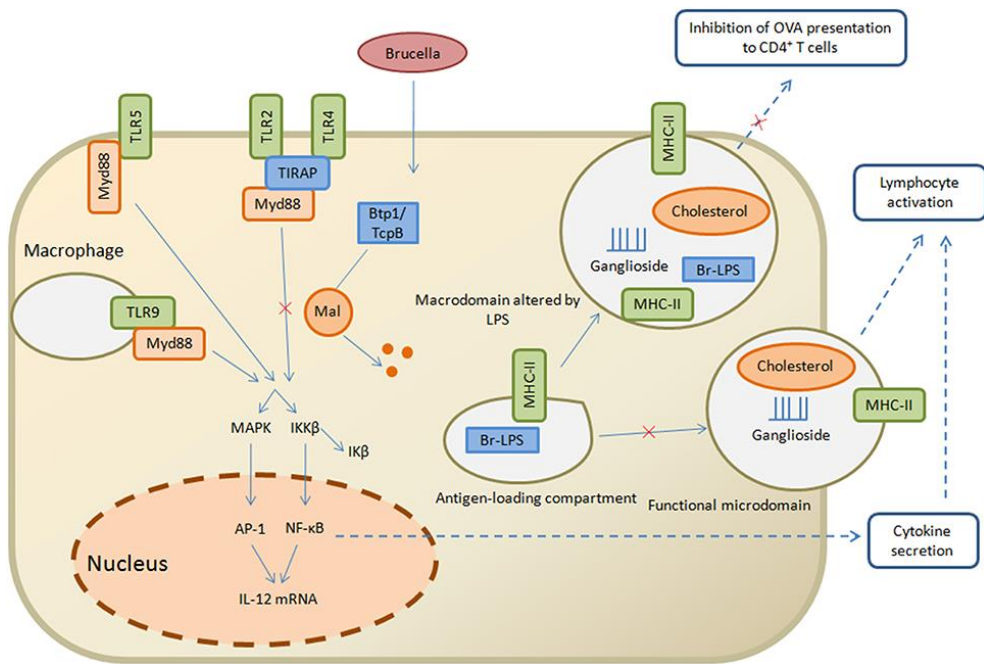


Figure 5. Strategies of *Brucella* to evade the innate and adaptive immunity.

(Adapted from Ahmed *et al.*, 2016).

Chapter I

Expression of Cytokine and Apoptosis-related Genes in Bovine Peripheral Blood Mononuclear Cells Stimulated with *Brucella abortus* Antigens

Abstract

Brucellosis is a clinically and economically important disease. Therefore, eradication programs of the disease have been implemented in several countries. One hurdle in these programs is the detection of infected animals at the early stage. Although the protein antigens as diagnostic antigens have recently received attention, the exact mechanisms at the beginning of immune responses are not yet known. Therefore, genes encoding five *Brucella abortus* cellular antigens were cloned and the expressed recombinant proteins were purified. The expression of several cytokine genes (IL-1 β , IL-4, IL-6, IL-12p40, IFN- γ , TNF- α , and iNOS) was analyzed in bovine peripheral blood mononuclear cells (bPBMC) after stimulation with *B. abortus* antigens. Three apoptosis-related genes, Bax, Bcl-2, and TLR4, were also included in the analysis to find out the adverse effects of the proteins to the cells.

Each protein induced different patterns of cytokine expression depending on the stimulation time and antigen dose. Expression of IL-6, IL-12p40, and IFN- γ was induced with all of the proteins while IL-1 β , IL-4, TNF- α , and iNOS gene expression was not. Expression of apoptosis-related genes was not altered except TLR4. These results suggest that the cellular antigens of *B. abortus* induce both humoral and cellular immunity via the production of IL-6, IL-12p40, and IFN- γ in bPBMC without exerting any adverse effects on the cells.

Key words: Brucellosis, *Brucella abortus* cellular antigen, Cytokines, bovine Peripheral mononuclear cell, Apoptosis-related gene.

Introduction

Brucellosis is a highly contagious zoonosis caused by Gram-negative bacteria of the genus *Brucella*. This disease affects livestock, wild animals, and humans. Ten species of the genus *Brucella* have been identified based on antigen variation and primary hosts [Corbel, 1997; Sung and Yoo, 2014]. Brucellosis causes significant economic losses not only because it affects animal production (reduced milk production, abortion, delayed conception, and impaired fertility) but also because detection of the disease in a region or country causes enactment of international veterinary regulations as well as restrictions on animal movements and trade [Corbel, 1997; Seleem *et al.*, 2010]. In addition, brucellosis in human can be severely debilitating and remains an important public health concern [Corbel, 1997; Young, 1995].

Most serological diagnostic methods for detecting *Brucella* infection use antibodies against common *Brucella* antigens [Nielsen and Yu, 2010]. O-polysaccharide (OPS), a well-known immune dominant epitope in smooth lipopolysaccharide (SLPS), is commonly used in serological tests for diagnosing brucellosis [Moreno *et al.*, 1984; Jiménez de Bagüés *et al.*, 1992; Gall *et al.*, 2008; McGiven, 2013]. Recently, several cellular proteins of *B. abortus* have been considered new diagnostic antigens because traditional diagnostic methods using *Brucella* LPS have low specificity due to cross-reactivity with other relevant bacteria such as *Yersinia enterocolitica* O:9 [Corbel, 1979; Nielsen *et al.*, 2004].

Brucella abortus is a facultative intracellular bacterial pathogen that can survive intracellular defenses and hamper the induction of host humoral immune responses [Cheers, 1984]. These properties help preventing the serological diagnosis of *B. abortus* infection. Infection with *B. abortus* potently activates both the innate and adaptive immune system, leading to a proinflammatory response that favors the T-helper 1 (Th1) responses [Dornand *et al.*, 2002; Golding *et al.*, 2001]. Although both antibody- and cell-mediated immune responses can influence the course of *Brucella* infection, the latter is primarily responsible for the clearance of intracellular bacteria [Li *et al.*, 2014].

Brucella spp. have mechanism that prevent activation of the host innate immune system [Barquero-Calvo *et al.*, 2007]. Invasion through the digestive tract does not elicit any inflammatory response including cytokine production from the host [Paixao *et al.*, 2009]. Therefore, *Brucella* spp. invade silently or unnoticed by the innate immune system of the host [Poester *et al.*, 2013].

Cytokines are important for responses to infection. Much attention has thus been given to research on cytokine-mediated inflammatory reactions in cases of brucellosis. Previous studies have revealed that *B. abortus* can induce the production of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-12 and IL-1 β by a variety of cell types and in mice [Zhan *et al.*, 1993; Zhan and Cheers, 1995; Zaitseva *et al.*, 1996; Huang *et al.*, 2003; Cha *et al.*, 2010].

Understanding immune mechanisms is an important step for the development of new control measures including diagnostic antigen(s) since most of the antigens have

been selected based on reaction with antibodies without considering the immune responses in the host [Ko *et al.*, 2012; Kim *et al.*, 2014; Lee *et al.*, 2014; Lee *et al.*, 2015]. However, the precious mechanisms of *B. abortus* infection including the possible apoptotic activities have not been revealed in the expression of cytokines and apoptosis-related genes, yet. Therefore, to understand the mechanism underlying the immune responses to *B. abortus*, bovine peripheral blood mononuclear cells (bPBMC) were treated with five well-known immunoreactive *B. abortus* cellular proteins. Cytokine production and the expression of genes associated with apoptosis were then analyzed as the first step of understanding in the induction of immune responses.

Materials and Methods

Cloning and expression of *B. abortus* genes

Genes encoding outer membrane protein 28 (OMP28), malate dehydrogenase (Mdh), elongation factor Ts (Tsf), arginase (RocF), and metal-dependent hydrolase (0628) of *B. abortus* 544 were amplified by PCR (Table 2), cloned, and expressed with a cold shock expression vector (pColdTM TF DNA; Takara, Japan) in *E. coli* DH5 α . Sequences of the primers used for this procedure are shown in Table 2. Nucleotide sequences of the genes were confirmed by sequencing using an automatic sequencer and dye-termination sequencing system.

Purification of the recombinant proteins

Five *E. coli* clones were cultured at 37 °C overnight in 100 ml of LB broth (Duchefa Biochemie, The Netherlands) with 10 µg of ampicillin (Duchefa Biochemie, The Netherlands). Sixty ml of the cultures were used to inoculate 1 liter of LB broth containing 100 µg of ampicillin. After culturing with shaking at 220 rpm for 3 h at 37 °C, isopropyl β-D-1-thiogalactopyranoside (IPTG; Amresco, USA) was added at a final concentration of 0.5 mM and the culture was further incubated overnight at 37 °C in a shaking incubator (Vision Science Co. Ltd., Korea) at 220 rpm. After incubation, the bacterial cells were harvested by centrifugation at 4400 x *g* for 20 min. The resulting pellets were resuspended in 40 mL of binding buffer (20 mM Tris–HCl, 8 M urea, 500 mM NaCl, 20 mM imidazole (Sigma, USA) [pH 8.0], and 1 mM β-mercaptoethanol (Sigma, USA) and sonicated at 10 000 Hz in ice water (60 % pulse, 20 s pulse/50 s steps, 15 cycles). Supernatants were collected after centrifugation at 4,400 x *g* for 20 min. Recombinant proteins were collected using a His-Spin Trap (GE Healthcare, UK) according to the manufacturer's protocol. Concentration of the purified recombinant proteins was measured using a BCA kit (Bio-Rad, USA). The recombinant proteins were analyzed by SDS-PAGE and Western blotting with an anti-His antibody (April Bio Co. Ltd., Korea). LPS contamination in the purified proteins was confirmed by endotoxin assay kit (Toxin Sensor™ Chromogenic LAL endotoxin Assay Kit, GenScript).

bPBMC isolation and culturing

Blood was collected into conical tubes (Nunc, USA) containing heparin from the jugular vein of clinically healthy Korean native cattle. The cattle were naïve to *B. abortus* exposure. The blood was overlaid onto 15 ml of Histopaque 1077 (Sigma, USA) in a 50 ml conical tube and centrifuged 400 x g for 30 min. Next, bPBMC were collected and washed three times with RPMI 1640 medium (Gibco, USA) containing 10 % fetal bovine serum (Gibco, USA). The bPBMC concentration was adjusted to 5×10^6 cells/ml and the cells were cultured in a 6-well plate with RPMI 1640 medium containing 10 % FBS for 8 h at 37 °C in a 5 % CO₂ atmosphere. After incubation, the bPBMC were stimulated with 5 or 10 µg/ml of the five different recombinant proteins for the indicated time intervals in the figures. LPS was removed by treatment with polymyxin B (10 µg/ml) before stimulation with the proteins. Concanavalin A (ConA, 1 µg/ml) was used as a positive control. All animal procedures were carried out according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Animal and Plant Quarantine Agency (South Korea). The study protocol was approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-IACUC: SNU-130916-3).

Purification of total RNA from bPBMC

Total RNA was isolated from the bPBMC using an RNeasy mini kit (Qiagen, Germany) according to the manufacturer's protocol. Before reverse transcription of the RNA using a QuantiTect® Reverse Transcription Kit (Qiagen, Germany) was

performed, genomic DNA was eliminated with 2 µl of gDNA wipeout buffer (7X), 11 µl of RNase-free water, and 1 µg of template RNA. After incubating the mixture at 42 °C for 2 min, reverse transcription was carried out with 1 µl of quantiscript reverse transcriptase, 4 µl of quantiscript RT buffer (5X), 1 µl of reverse transcription primer mix, and 14 µl of cDNA generated by incubation at 42 °C for 15 min. Quantiscript reverse transcriptase was inactivated by incubation at 95 °C for 15 min. The reaction products were used to analyze gene expression with real-time PCR.

Analysis of cytokine and apoptosis-associated genes by real-time PCR

Real-time PCR was carried out using the cDNA products after completing reverse transcription-PCR. A Rotor-Gene SYBR Green PCR Kit (Qiagen, Germany) was used with a two-step cycling protocol including denaturation at 95 °C and a combined annealing/extension step dependent upon the primer T_m value according to the manufacturer's protocol. The reaction mixture contained 10 µl of 2X Rotor-Gene SYBR Green PCR Master Mix, 2 µl of the primers, 2 µl of template DNA or cDNA, and 6 µl of RNase-free water. The real-time PCR conditions were as follows: initial denaturation at 95 °C for 5 min followed by 50 cycles of 95 °C for 20 s and annealing at 55 °C or 60 °C for 10 s. The expression of each gene was normalized relative to the expression of β-actin. Genes encoding inducible nitric oxide synthase (iNOS), IL-1β, IL-4, IL-6, IL-12p40, interferon (IFN)-γ, and TNF-α were analyzed. Three apoptosis-related genes, Bcl-2-associated X protein (Bax), B cell lymphoma

2 (Bcl-2), and Toll-like receptor-4 (TLR4), were also analyzed. Sequences of the primers and probes used in this real-time PCR are presented in Table 3.

Statistical analysis

Data are reported as the mean \pm standard error of the mean (SEM) of three or more independent experiments. Statistically significant was determined by the Student *t* test using statistical package for social science (SPSS) software version 21. Differences were considered to be significant if a *P* value was < 0.05 .

Results

Genes encoding five different cellular proteins of *B. abortus* (rOMP28, rMdh, rTsf, rRocF, and r0628) were cloned, sequenced, and expressed in *E. coli*. The expressed proteins were purified and analyzed by SDS-PAGE and Western blotting using an anti-His antibody (Figure 6). As a result of LPS contamination, treatment of PBMC with each *B. abortus* antigens showed 0.01 EU/ml (measurement range 0.01 - 1 EU/ml), and the intracellular effects due to LPS contamination were negligible after confirmed the number of endotoxin unit. Generally, treatment with each recombinant protein induced different patterns of cytokine expression depending on stimulation time and dose. However, the expression of apoptosis-related genes was not greatly affected by stimulation with *B. abortus* antigens. Induction of iNOS gene expression

was up-regulated at 12 or 24 h after stimulation of bPBMC with 10 µg of rOMP28, rMdh, rTsf, and rRocF proteins ($P < 0.01$) while there was no significant change in the gene expression with 5 µg of all *B. abortus* antigens (Figure 7). IL-1 β gene expression was significantly down-regulated dose and time-dependently after stimulation with 5 µg and 10 µg of all *B. abortus* antigens in the cells ($P < 0.05$) (Figure 8). In case of IL-4, induction of significant gene expression was observed in the bPBMC stimulated with only 5 µg of rRocF protein ($P < 0.05$) even though 10 µg of rTsf and r0628 proteins induced the gene expression in the cells (Figure 9). Expression of IL-6 gene was highly induced time and dose-dependently with all *B. abortus* antigens ($P < 0.01$) (Figure 10). rMdh and rRocF were the most effective inducers in the IL-6 gene expression in the bPBMC ($P < 0.01$). Also, 10 µg of rTsf induced the higher gene expression of IL-6 at 12 h after the stimulation ($P < 0.01$). Induction of IL-12p40 gene expression was the most effective when the cells were stimulated with 5 µg of all *B. abortus* antigens for 12 h ($P < 0.01$) (Figure 11). The induction was also effective with 10 µg of rOMP28 and r0628 proteins at 12 and/or 24 h after the stimulation ($P < 0.01$). IFN- γ gene expression was significantly induced in the cells stimulated with 5 µg of all *B. abortus* antigens for 12 h ($P < 0.01$) even though the significant induction was also observed with 10 µg of rOMP28 and r0628 proteins ($P < 0.01$) (Figure 12). After stimulation of the cells with 10 µg of all *B. abortus* antigens, TNF- α gene expression was significantly induced at 12 and/or 24 h ($P < 0.01$). Five µg of *B. abortus* antigens could not induce the TNF- α gene expression except at 24 h with rTsf protein (Figure 13).

In apoptosis related gene expression, Bax, Bcl-2 and TLR4, the gene expression was mostly down-regulated in the cells stimulated with 5 µg of all *B. abortus* antigens even though 10 µg of some *B. abortus* antigens induced the gene expression at different times (Figures 14, 15, 16). The gene expression of Bax was down-regulated with 5 µg of *B. abortus* antigens ($P < 0.05$) while 10 µg of rOMP28, rRocF and r0628 proteins up-regulated the gene expression ($P < 0.05$) (Figure 14). However, the changes might not be effective even though there was significant difference. Bcl-2 gene expression was effectively up-regulated with 10 µg of rMdh in 24 h stimulation ($P < 0.01$). There were no meaningful changes in the gene expression of Bcl-2 (Figure 15). TLR4 gene expression was significantly down-regulated when the cells stimulated with 5 µg of all *B. abortus* antigens ($P < 0.05$) while 10 µg of rMdh and rRocF proteins induced higher gene expression of TLR4 ($P < 0.05$) (Figure 16).

In summary, the production of iNOS, IL-4 and TNF- α was not effectively induced in bPBMC by stimulation with *B. abortus* antigens even though some induction was observed at certain times by treatment with 10 µg of rOMP28, rTsf, and rRocF. IL-1 β gene expression was down-regulated in dose and time-dependent manner in the stimulation of cells with all *B. abortus* antigens. IL-6, IL-12p40 and IFN- γ gene expression was effectively induced in the cells stimulated with all *B. abortus* antigens, especially 5 µg of *B. abortus* antigens. The expression of a gene associated with apoptosis in bPBMC was not induced by exposure to 5 µg of the *B. abortus* antigens. However, expression of the apoptotic genes was induced with different

expression profiles at 12 or 24 h by stimulation with 10 µg of some *B. abortus* antigens.

Discussion

Brucellosis is a re-emerging zoonosis that has regained attention of the scientific community because pathogenesis of this disease in humans and animals has significantly evolved [Seleem *et al.*, 2010; Pappas, 2010]. However, the overall burden of the disease remains underestimated and has not been well studied. Eradication of brucellosis in animals is important for prevention of this disease in humans and requires optimal diagnosis along with vaccination [McGiven *et al.*, 2006]. The cellular proteins of *B. abortus* have received increased attention in the development of diagnostic techniques and vaccines given the important roles of these proteins in the early stage of infection [Cha *et al.*, 2010]. In addition, the information will help to reveal mechanisms underlying the pathogenesis of *Brucella* infection. *Brucella* is able to infect macrophages, and persist and replicate in the intracellular environment [Hamer *et al.*, 2014]. Identifying bacterial proteins that are necessary for intracellular survival of *Brucella* may provide new insights into mechanisms associated with pathogenesis and immune protection along with candidate antigens for diagnosis and vaccines [Goldbaum *et al.*, 1993; Baldi *et al.*, 1996; Ghasemi *et al.*, 2014]. Although several immunogenic proteins of *B. abortus* have been

identified [Ko *et al.*, 2012; Kim *et al.*, 2014; Lee *et al.*, 2014; Lee *et al.*, 2015], roles of these proteins still remain unclear. Based on recently acquired knowledge, five different cellular proteins of *B. abortus* with unknown potential in the induction of immune responses were selected for analysis in this study and effects of *B. abortus* antigens on bPBMC were investigated.

To evaluate the potential of five *B. abortus* antigens as diagnostic antigens, underlying mechanisms of the proteins in PBMC were investigated. Therefore, five genes were cloned, and the recombinant proteins were expressed and purified. After stimulation of bPBMC with *B. abortus* antigens, the expression of cytokines and apoptosis-related genes were analyzed by real-time reverse transcription-PCR. Most of the proteins induced the expression of IL-6, IL-12p40 and IFN- γ in a time- and dose-dependent manner. This result concurs with finding from a previous study showing that high amounts of IFN- γ , IL-12, and IL-6 are produced by splenocytes of mice vaccinated with chaperone protein DnaK [Ghasemi *et al.*, 2014]. In vivo-induced antigen technology (IVIAT) using elk (*Cervus elaphus*) revealed that Mdh is a predictor of natural infection [Lowry *et al.*, 2010]. Mdh is commonly expressed during infection in cattle and elk, and is not only an immunogenic protein but also promotes bacterial pathogenesis as a new virulence factor [Lowry *et al.*, 2010; Han *et al.*, 2014]. These data are similar to our results showing the effect of Mdh on cytokine production.

The *B. abortus efp* mutant has slower growth in complex media and higher sensitivity to detergents [Iannino *et al.*, 2012]. The *efp* gene is also required for

internalization in nonprofessional macrophages and HeLa cells [Iannino *et al.*, 2012]. However, the gene does not appear to be associated with virulence in professional macrophages, J774 macrophage-like cells, or mice [Iannino *et al.*, 2012].

Immune responses rely to a great extent on the recognition of foreign antigens by toll-like receptors (TLR). These receptors detect different pathogen-associated molecular patterns (PAMP), and trigger the activation of myeloid differentiation primary response gene 88 (MyD88)- and TIR-domain-containing adaptor-inducing interferon- β (TRIF)-dependent signaling pathways. This in turn leads to a wide range of cellular responses including the secretion of proinflammatory cytokines, chemokines, and type I interferons [Kawai and Akira, 2010]. In this study, gene expression of TLR4 was down-regulated up to 24 h and up-regulated in the stimulation with *B. abortus* antigens. Previous studies on the TLR4 expression showed down-regulation of TLR4 expression up to 24 h and upregulation of the expression after 24 h in the macrophage cells stimulated *B. abortus* or LPS of the bacterium [Weiss *et al.*, 2005; Nomura *et al.*, 2000]. This phenomenon was similar with this study with bPBMC.

These results suggest that *B. abortus* antigens might induce proper immune responses without adverse effects (such as apoptosis) in the bPBMC. Gene expression of iNOS, TNF- α , and IL-1 β was not effective in the cells by stimulation with *B. abortus* antigens. On the contrary, gene expression of IL-6, IL-12p40, and IFN- γ was significantly increased with *B. abortus* antigens. These results indicate that the adaptive immune systems are effectively activated by *B. abortus* antigens in

bPBMC. In conclusion, the five *B. abortus* antigens examined in this study appear to induce effective adaptive immune responses in both humoral and cellular immunity without induction of inflammatory reaction.

Table 2. Primer sequences for cloning *Brucella abortus* genes

Gene	Primer sequence (5' - 3')	Annealing temperature (°C)	Product size (bp)	Reference
Outer membrane protein 28 (OMP28)	F: GATCGGATCCAACACTCGTGCTAGCAATTTT R: GATCAAGCTTTTACTTGATTCAAAAACGAC	60	482	[Im <i>et al.</i> , 2016a]
Malate dehydrogenase (Mdh)	F: AATTCGGATCCATGGCACGCAACAAGATT R: AGGCGTCGACTTATTTTCAGCGACGGAGC	55	563	[Im <i>et al.</i> , 2016a]
Elongation factor (Tsf)	F: AATTCGAATTCATGAGCATTTCGCGCATCT R: AGGCCTGCAGTCAGCCCTTGGCGGCTGCGGC	55	314	[Im <i>et al.</i> , 2016a]
Arginase (RocF)	F: AGCGCGGATCCATGCATTGTAAGATTCTG R: AGGCCTGCAGTCAATAGCTGATGGTCGG	55	572	[Im <i>et al.</i> , 2016a]
Metal-dependent hydrolase (0628)	F: AGCGCGGATCCATGCATTGTAAGATTCTG R: AGCGCTGCAGTTAAGCTTGGAAGCTGTG	55	624	[Im <i>et al.</i> , 2016a]

Table 3. The primer sets for real-time PCR

Gene	Primer sequence (5' - 3')	Annealing temperature (°C)	Product size (bp)	Reference
iNOS	F: AGCGGAGTGACTTTCCAAGA R: TTTTGGGGTTCATGATGGAT	55	97	[Okagawa <i>et al.</i> , 2012]
IL-1 β	F: ACCTTCATTGCCCAGGTTTCT R: TGTTTGGGGTCATCAGCCTCAA	55	120	[Okagawa <i>et al.</i> , 2012]
IL-4	F: CAAAGAACACAACATAAGAAG R: AGGTCTTTCAGCGTACTTGT	55	181	[Okagawa <i>et al.</i> , 2012]
IL-6	F: TCCAGAATGAGTATGAGG R: CATCCGAATAGCTCTCAG	55	236	[Okagawa <i>et al.</i> , 2012]
IL-12p40	F: AACCTGCAACTGAGACCATT R: ATCCTTGTGGCATGTGACTT	55	186	[Okagawa <i>et al.</i> , 2012]
IFN- γ	F: ATAACCAGGTCATTCAAAGG R: ATTCTGACTTCTCTTCCGCT	55	218	[Okagawa <i>et al.</i> , 2012]
TNF- α	F: TAACAAGCCAGTAGCCACG R: GCAAGGGCTCTTGATGGCAGA	55	277	[Okagawa <i>et al.</i> , 2012]
Bax	F: TCTCCCCGAGAGGTCTTTTT R: TGATGGTCCTGATCAACTCG	55	151	[Xu <i>et al.</i> , 2012]
Bcl2	F: ATGTGTGTGGAGAGCGTCAA R: CTAGGGCCATACAGCTCCAC	55	146	[Xu <i>et al.</i> , 2012]
TLR4	F: TGACATCTTCACAGAACTGACTA R: GGAGTGGTTCATAAAGAAATGTA	55	164	[Im <i>et al.</i> , 2016a]
β -actin	F: CGCACCCTGGCATTGTCAT R: TCCAAGGCGACGTAGCAGAG	60	227	[Konnai <i>et al.</i> , 2003]

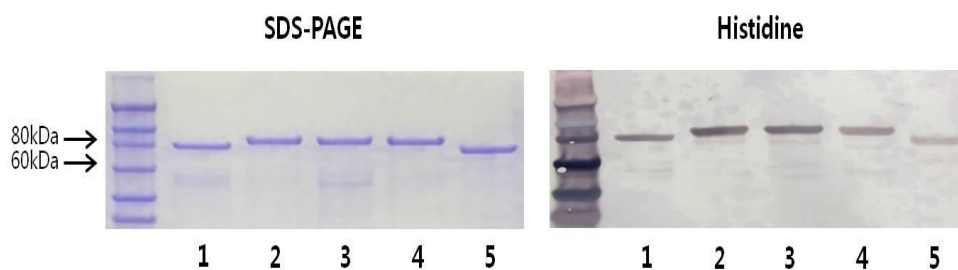


Figure 6. Analysis of the purified recombinant proteins. SDS-PAGE and Western blotting with an anti-His antibody. Lane M: molecular weight markers (Life Technologies, USA), lane 1: Outer membrane protein 28 (OMP28) (88 kDa), lane 2: Malate dehydrogenase (Mdh) (91 kDa), lane 3: Elongation factor (Tsf) (93 kDa), lane 4: Arginase (RocF) (91 kDa), lane 5: Metal-dependent hydrolase (0628) (85 kDa).

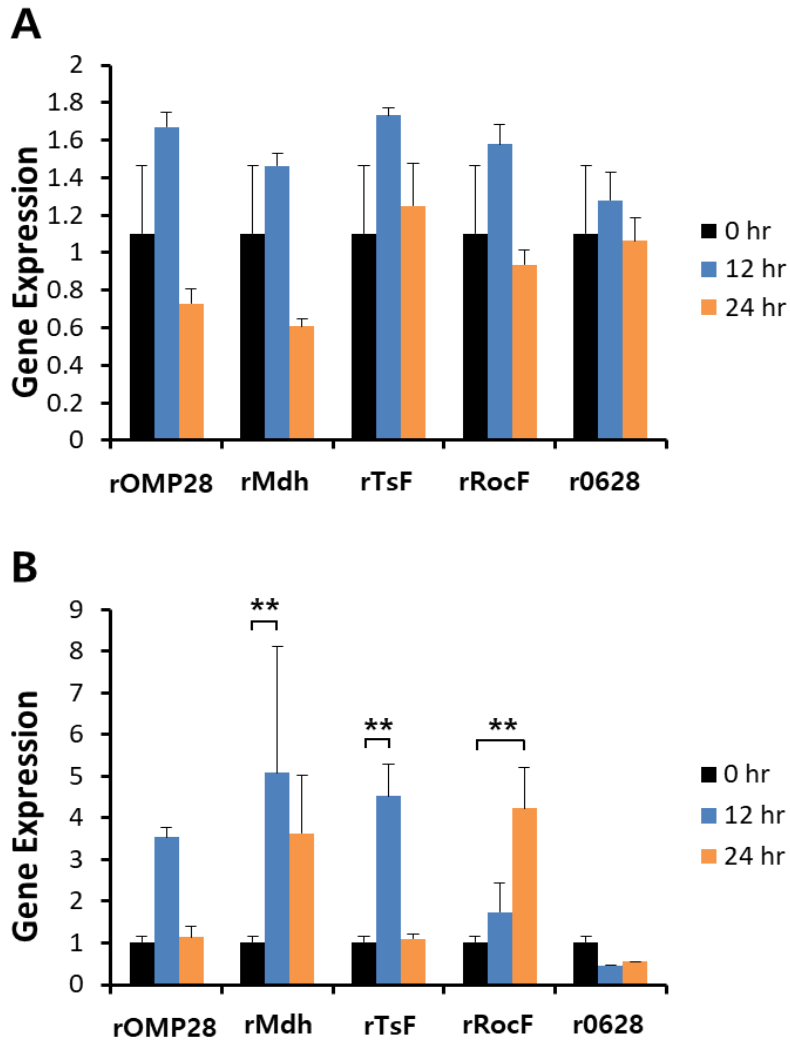


Figure 7. Gene expression of inducible nitric oxide synthase (iNOS) in bovine PBMC. Bovine PBMC were stimulated with 5 µg (A) and 10 µg (B) of five different cellular antigens (rOMP28, rMdh, rTsF, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β -actin. (** $P < 0.01$).

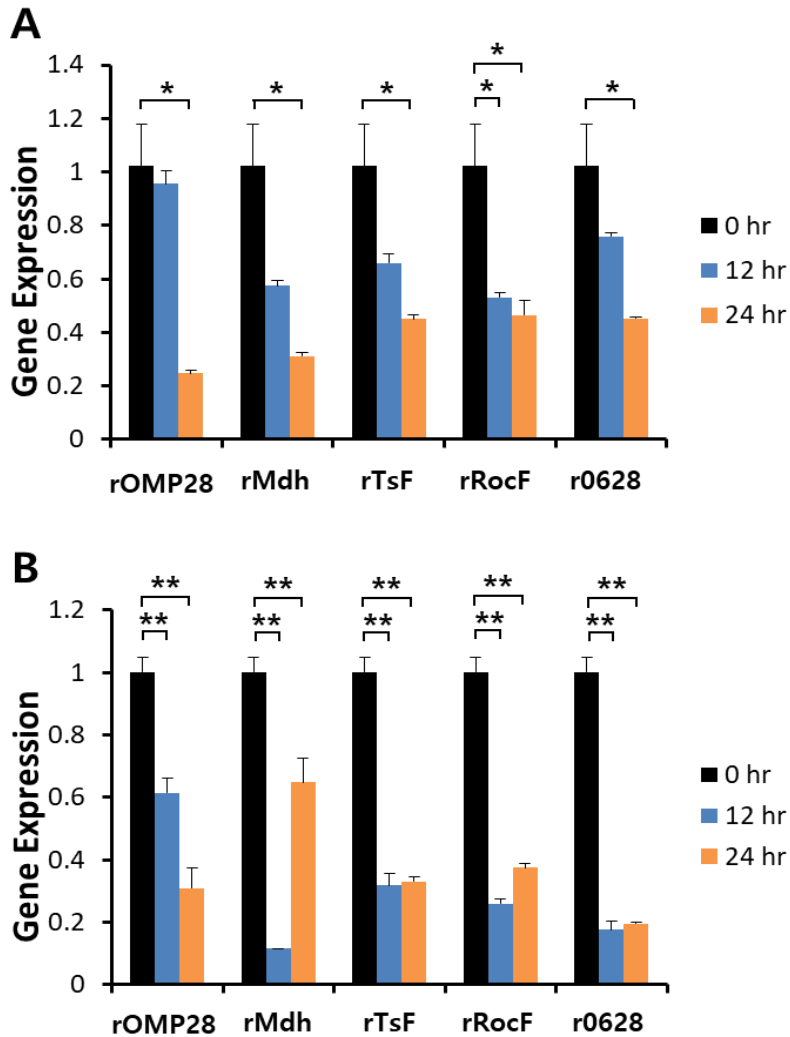


Figure 8. Gene expression of interleukin-1 β (IL-1 β) in bovine PBMC. Bovine PBMC were stimulated with 5 μ g (A) and 10 μ g (B) of five different cellular antigens (rOMP28, rMdh, rTsF, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β -actin. (* $P < 0.05$, ** $P < 0.01$).

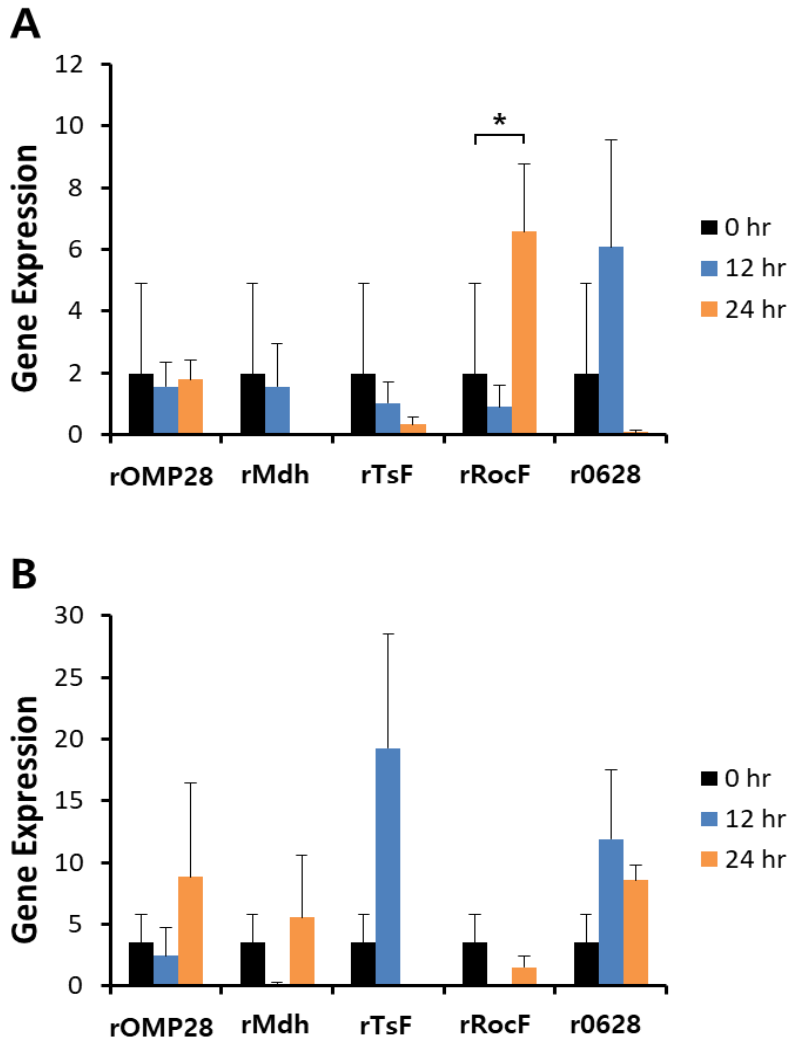


Figure 9. Gene expression of interleukin-4 (IL-4) in bovine PBMC. Bovine PBMC were stimulated with 5 µg (A) and 10 µg (B) of five different cellular antigens (rOMP28, rMdh, rTsF, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β -actin. (* $P < 0.05$).

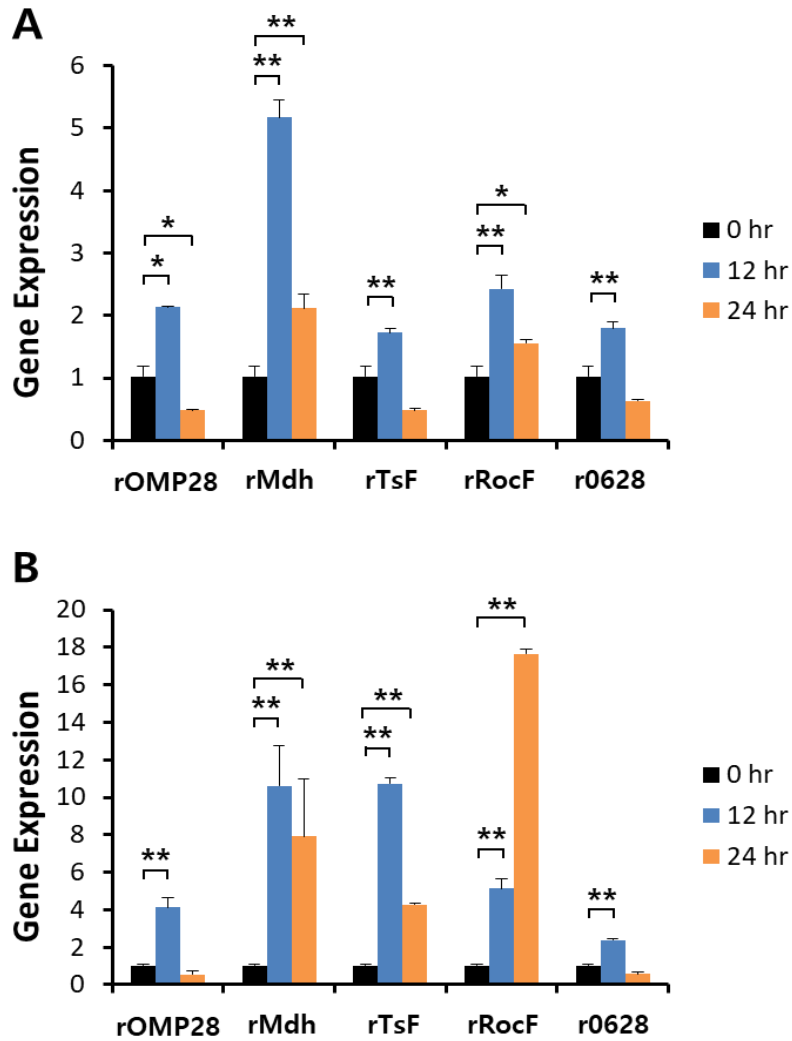


Figure 10. Gene expression of interleukin-6 (IL-6) in bovine PBMC. Bovine PBMC were stimulated with 5 µg (A) and 10 µg (B) of five different cellular antigens (rOMP28, rMdh, rTsF, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β-actin. (* $P < 0.05$, ** $P < 0.01$).

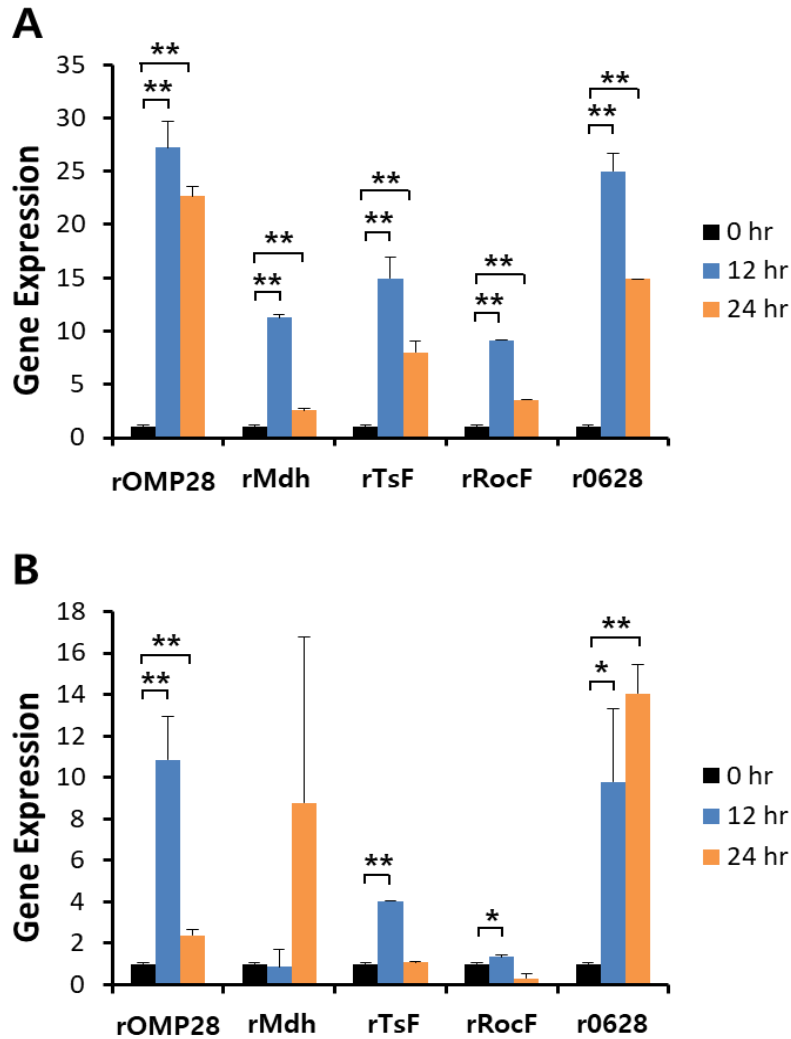


Figure 11. Gene expression of interleukin-12p40 (IL-12p40) in bovine PBMC. Bovine PBMC were stimulated with 5 µg (A) and 10 µg (B) of five different cellular antigens (rOMP28, rMdh, rTsf, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β -actin. (* $P < 0.05$, ** $P < 0.01$).

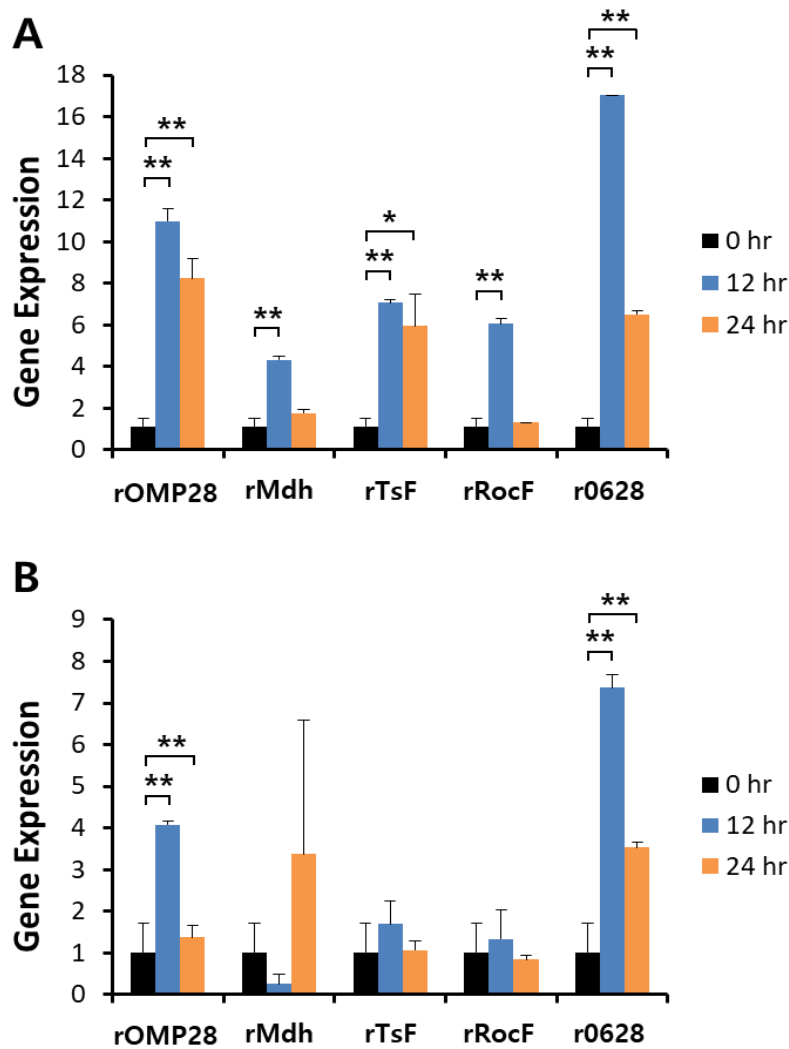


Figure 12. Gene expression of interferon- γ (IFN- γ) in bovine PBMC. Bovine PBMC were stimulated with 5 μ g (A) and 10 μ g (B) of five different cellular antigens (rOMP28, rMdh, rTsF, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β -actin. (* $P < 0.05$, ** $P < 0.01$).

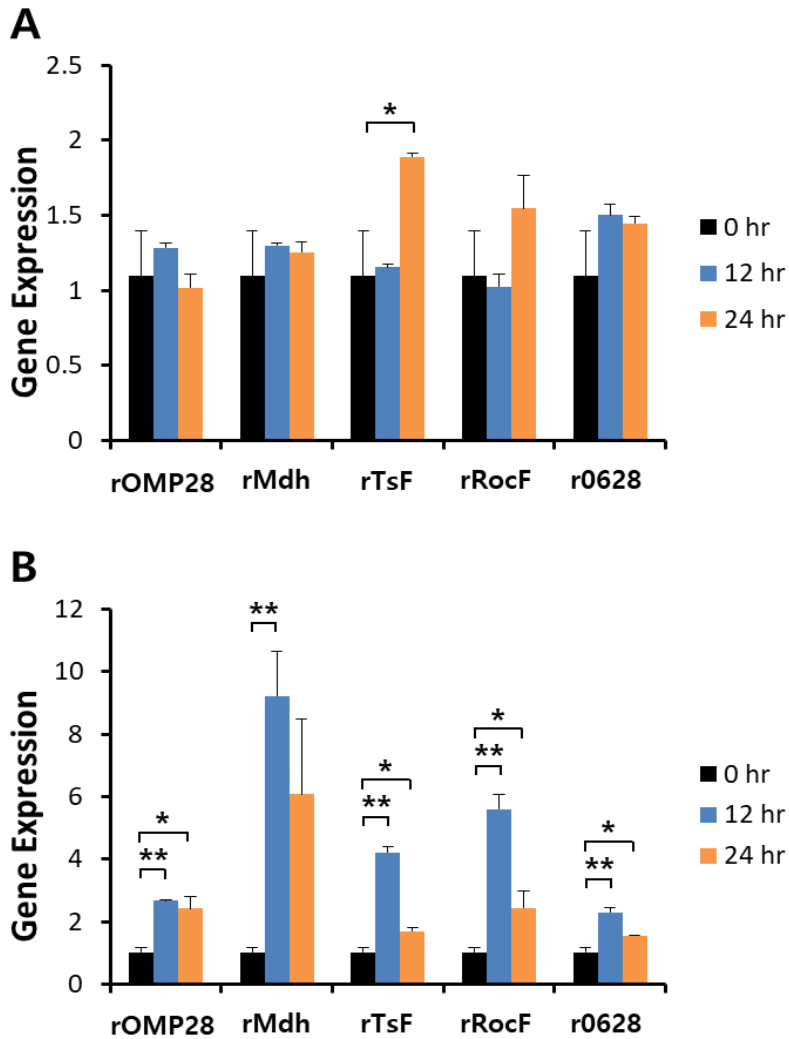


Figure 13. Gene expression of tumor necrosis factor- α (TNF- α) in bovine PBMC. Bovine PBMC were stimulated with 5 μ g (A) and 10 μ g (B) of five different cellular antigens (rOMP28, rMdh, rTsF, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β -actin. (* $P < 0.05$, ** $P < 0.01$).

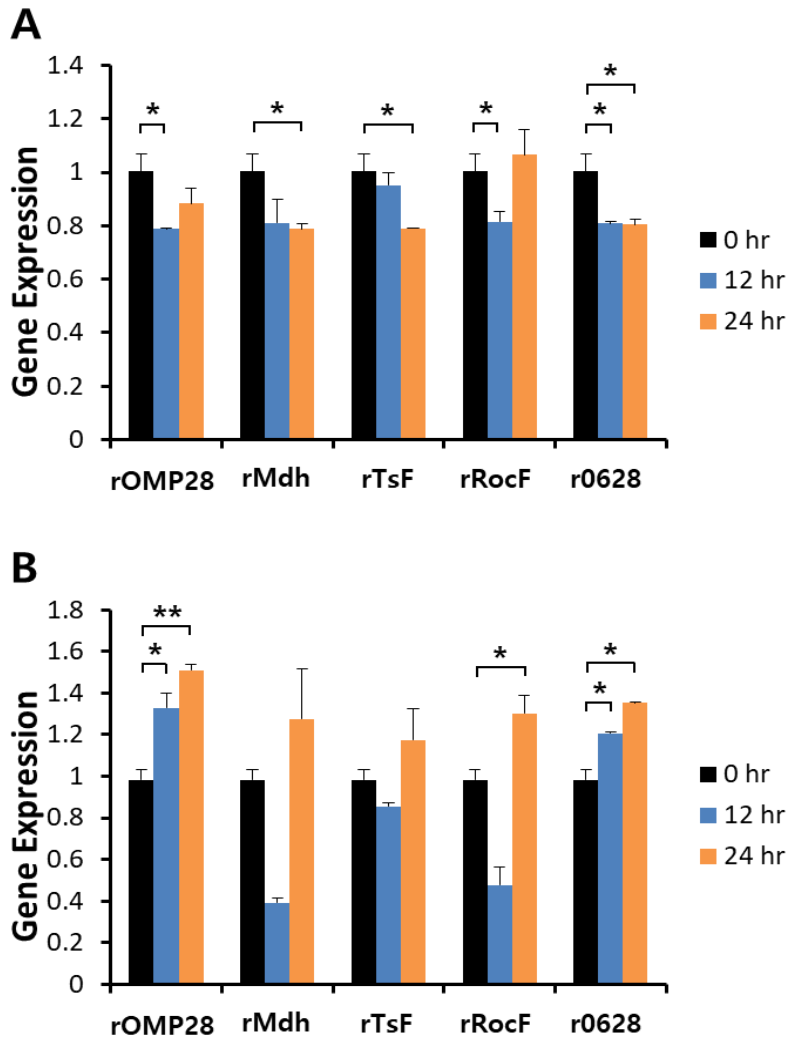


Figure 14. Gene expression of Bax in bovine PBMC. Bovine PBMC were stimulated with 5 µg (A) and 10 µg (B) of five different cellular antigens (rOMP28, rMdh, rTsf, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β -actin. (* $P < 0.05$, ** $P < 0.01$).

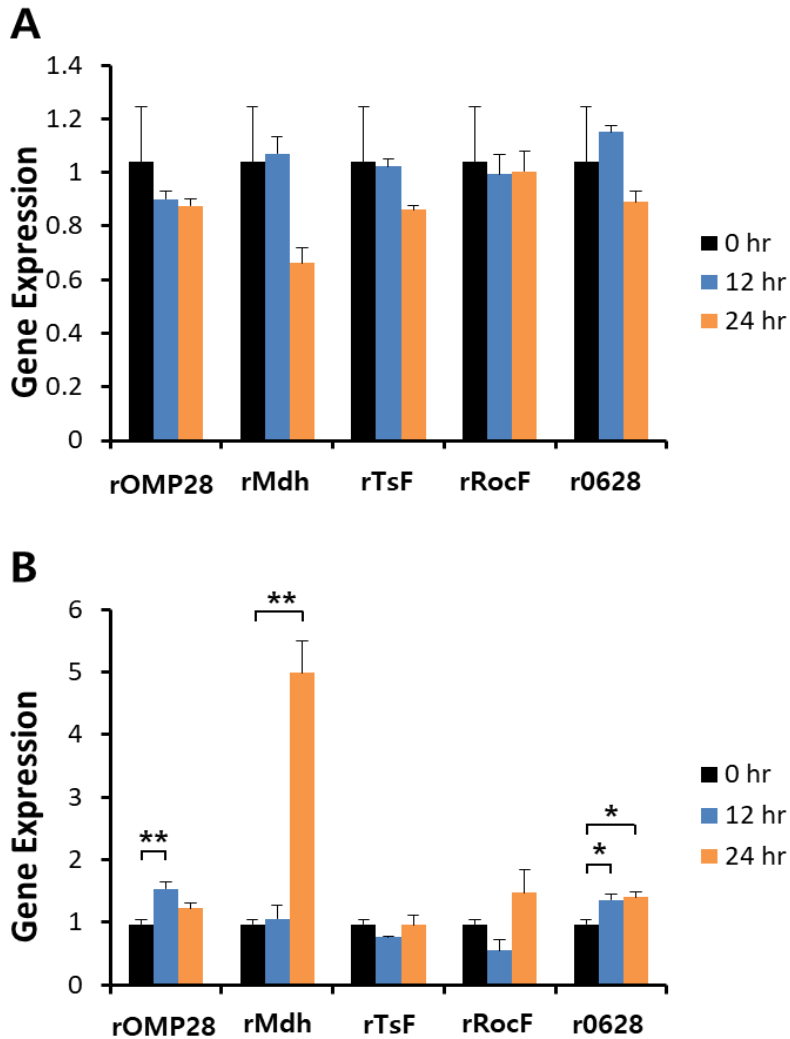


Figure 15. Gene expression of Bcl-2 in bovine PBMC. Bovine PBMC were stimulated with 5 µg (A) and 10 µg (B) of five different cellular antigens (rOMP28, rMdh, rTsf, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β -actin. (* $P < 0.05$, ** $P < 0.01$).

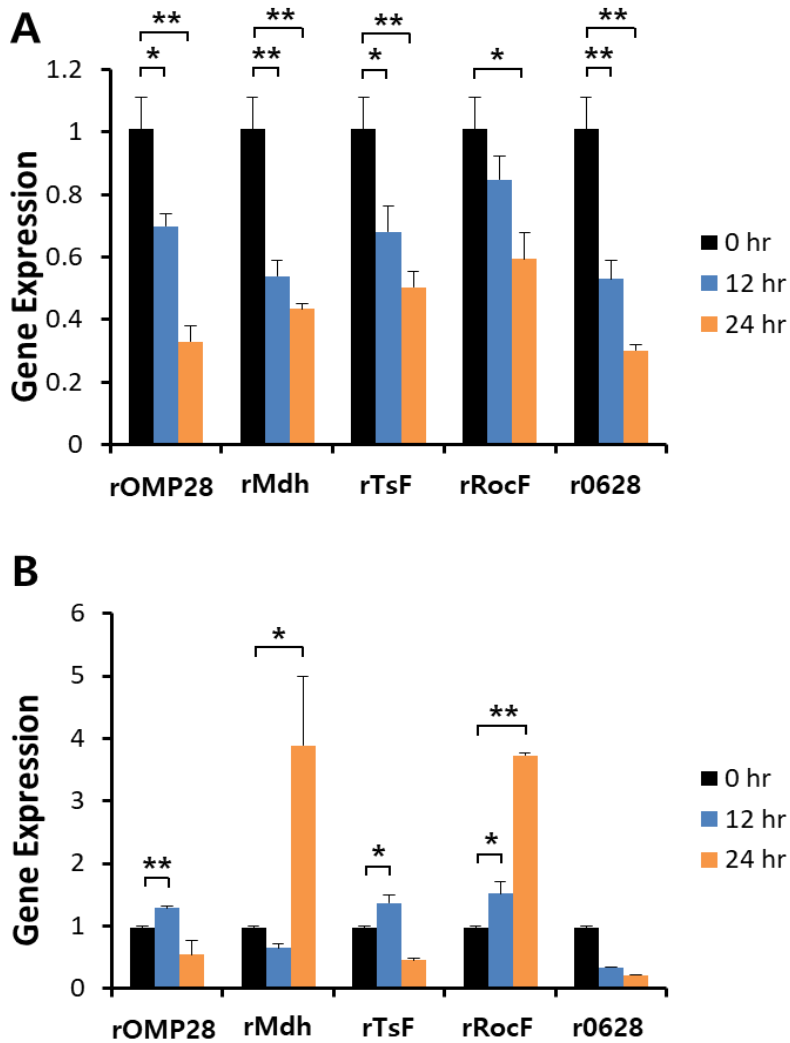


Figure 16. Gene expression of Toll-like receptor 4 (TLR4) in bovine PBMC. Bovine PBMC were stimulated with 5 µg (A) and 10 µg (B) of five different cellular antigens (rOMP28, rMdh, rTsF, rRocF, and r0628) of *Brucella abortus* at 0, 12, and 24 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of β-actin. (* $P < 0.05$, ** $P < 0.01$).

Chapter II

Th2-related Immune Responses in *in vitro* and *in vivo* Stimulated with *Brucella abortus* Antigens, Malate dehydrogenase (Mdh), Elongation factor (Tsf) and Arginase (RocF)

Abstract

Brucellosis is an important zoonotic disease caused by *Brucella* species. The disease is difficult to control due to the intracellular survival of the bacterium and the lack of precise understanding of pathogenesis. Despite of continuous researches on the pathogenesis of *Brucella* spp. infection, there is still question on the pathogenesis, especially earlier immune response in the bacterial infection. Recombinant proteins, Malate dehydrogenase (rMdh), elongation factor (rTsf), and arginase (rRocF), which showed serological reactivity, were purified after gene cloning, and their immune modulating activities were then analyzed in a murine model. Cytokine production profiles were investigated by stimulating RAW 264.7

cells and naïve splenocytes with the three cellular antigens of *Brucella abortus*. Also, immune responses were analyzed by ELISA and an ELISpot assay after immunizing mice with the three proteins. Only TNF- α was produced in stimulated RAW 264.7 cells, whereas Th1-related cytokines, IFN- γ and IL-2, were induced in naïve splenocytes. In contrast, Th2-type immune response was more strongly induced in antigen-secreting cells in the splenocytes obtained 28 days after immunizing mice with the three *B. abortus* antigens, as were IgM and IgG. The induction of Th2-related antibody, IgG1, was higher than the Th1-related antibody, IgG2a, in immunized mice. These results suggest that the three *B. abortus* antigens strongly induce Th2-type immune response *in vivo*, even though Th1-related cytokines were produced *in vitro*.

Keywords: *Brucella abortus*, antigens, Th1, Th2, Immune responses.

Introduction

Brucella spp. causes brucellosis, a major zoonotic disease, and is a facultative intracellular bacterium that might induce complicated immune responses. Brucellosis is considered as an important disease in both clinically and economically. Eleven species have been classified based on primary preferred host and antigenic variation : *B. melitensis* (sheep and goats), *B. suis* (hogs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats), *B. ceti* (seals), *B. pinnipedialis* (pinnipeds), *B. microti* (common vole), *B. inopinata* (breast implant infection), and *B. papionis* (baboon) [Ko and Splitter, 2003; Foster *et al.*, 2007; Scholz *et al.*, 2010; Whatmore *et al.*, 2014]. The intracellular survival and replication characteristics of *Brucella* spp. enable it to establish chronic infections in host cells [Dettileux *et al.*, 1990a; Dettileux *et al.*, 1990b].

B. abortus causes undulant fever, endocarditis, arthritis and osteomyelitis in man and abortion and infertility in cattle [Franco *et al.*, 2007]. It easily infects animals by ingestion or inhalation bacterium in reproductive tissues, fetal fluids, and mammary gland. In man, brucellosis is usually transmitted by direct or indirect contact with abortion related discharges and ingestion of unpasteurized dairy products from the infected animals. To control brucellosis in man and cattle, it is considered the identification and removal of infected animals and/or vaccination have been used depending on infection status of countries.

Lipopolysaccharide (LPS) has been considered to be the most important antigen

for the diagnosis of brucellosis although several other cellular antigens are involved in immune responses to the bacterium [Baldi *et al.*, 1996]. Therefore, LPS-based antigens have been used for the serological confirmation of *B. abortus* infection in ruminants. Indirect diagnosis approaches are most widely used, such as, serological tests based on LPS, the standard tube agglutination test, the Rose Bengal plate agglutination test, the complement fixation test, and enzyme-linked immunosorbent assays. Although LPS elicits strong immune response, LPS-based diagnostic methods have several problems, such as, difficult differentiation of infected and vaccinated animals and cross-reactivity with various Gram-negative bacteria, like *Yersinia enterocolitica* O:9, *Escherichia coli*, *Salmonella enterica* serotype Urbana [Kittelberger *et al.*, 1998; Oñate *et al.*, 1999; Ruiz-Mesa *et al.*, 2005; Nielsen *et al.*, 2007]. Therefore, many researchers have tried to develop non-LPS based diagnostic tools [Al Dahouk *et al.*, 2006].

Recently, specific immune-reactive proteins of *B. abortus* were identified during proteomic analyses [Al Dahouk *et al.*, 2006; Connolly *et al.*, 2006; Ko *et al.*, 2012; Lim *et al.*, 2012a; Ghasemi *et al.*, 2014]. Outer membrane proteins (OMPs) are considered major immunoreactive components of bacterial cells to develop new diagnostic or vaccine candidates [Cloeckaert *et al.*, 1991; Chaudhuri *et al.*, 2010; Ghasemi *et al.*, 2014; Simborio *et al.*, 2015], and many researchers working on the development of new diagnostic markers for brucellosis have studied other recombinant proteins of *Brucella*. In addition, immunodominant antigens have been investigated after immunization with *Brucella* spp. [Sung *et al.*, 2014; Lee *et al.*,

2015]. Most microorganisms have developed a transport and storage system to enhance survivability. In particular, iron is an essential element, and the role of bacterioferritin (Bfr) has been studied in the contexts of iron metabolism and survival in macrophages [Denoel *et al.*, 1997]. Additionally, Lee *et al.* reported that the levels of expression some proteins, such as, malate dehydrogenase (Mdh), elongation factor (Tsf), and arginase (RocF), were correlated with the pathogenesis of *Brucella* infections and the metabolism of *Brucella* [Lee *et al.*, 2015]. However, some mechanisms responsible for the immune responses of *Brucella* proteins have been studied but there are still insufficient about described immune mechanisms. To evaluate the induction of immunological responses after stimulation with three recombinant proteins, the production levels of T helper 1 (Th1)-related cytokines and T helper 2 (Th2)-related cytokines were measured. In addition, the induction of IgM, IgG1, G2a and total IgG were evaluated for determination of immune response types by measuring the number of antigen-secreting cells. In this study, three *B. abortus* antigens, Mdh, Tsf, and RocF, were investigated by examining production of cytokines and immune responses to cellular antigens of *B. abortus* *in vitro* and *in vivo* to determine their possible usages as protein antigens for the diagnosis of bovine brucellosis.

Materials and Methods

Bacterial strains and construction of Mdh, Tsf and RocF expression clones

Bacterial strains were cultured as previously described [Sung *et al.*, 2014; Im *et al.*, 2016b]. Total genomic DNA was prepared from *B. abortus* 544 culture using the G-spin[™] Genomic DNA Extraction kit for bacteria (Intron, Korea). Genes encoding Mdh, Tsf, and RocF of *B. abortus* were amplified by PCR using the primers pairs shown in Table 4. After digestion with appropriate restriction enzymes, amplified DNA was ligated into pCold TF vector (Takara, Japan). Ligated plasmids were then transformed into the *E. coli* DH5 α host cells. Conservation of insert sequences in the expression vector was confirmed by nucleotide sequencing.

Expression and purification of rMdh, rTsf and rRocF proteins

The expression and purification of recombinant proteins were modified as previously described [Sung *et al.*, 2014; Im *et al.*, 2016b]. In brief, 20 ml of overnight cultured *E. coli* harboring plasmids was inoculated into one liter of LB broth containing ampicillin (25 μ g/ml) and cultured at 37 °C for 7 h. Protein expression was then induced with isopropyl β -D-1-thiogalactopyranoside (Amresco, USA) at a final concentration of 0.3 mM. Cells were then incubated at 37 °C for 2 h and harvested by centrifugation at 4,400 x g for 20 min. Cell pellets were resuspended in 40 ml Column buffer (20 mM Tris HCl, 8 M Urea, 500 mM NaCl, 20 mM Imidazole, 1 mM β -mercaptoethanol, pH 8.0), sonicated at 10,000 Hz in an ice-water bath, centrifuging at 4,400 x g for 20 min. Supernatants were then loaded onto a His SpinTrap (GE Healthcare, UK) column in accordance with the manufacturer's

instructions. Purified proteins were stored at -20 °C until required.

SDS-PAGE and Western blot

Purified recombinant proteins were confirmed by SDS-PAGE and Western blotting. In brief, purified recombinant proteins diluted with sample buffer were denatured by boiling for 10 min at 100 °C, electrophoresed, and visualized using Coomassie Brilliant Blue R-250 (Intron, Korea). For Western blot, proteins were resolved by SDS-PAGE and electro-transferred to nitrocellulose membranes (Invitrogen, USA) for 25 min using an iBlot® transfer device (Invitrogen, USA). Membranes were incubated in 5 % skim milk (BD, USA) solution for 1 h at room temperature (RT), and washed 3 times with washing buffer (TBS). Proteins were probed with anti-histidine (1:2,000 dilution, AprilBio Co., Ltd, Korea) by incubation for 3 h at RT. Unbounded antibodies were removed by washing (3 x 10 minutes) with the same buffer. Membranes were then incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (1:2,000; Bethyl, USA,) for 1 h at RT. After a final wash, proteins were visualized using the AP conjugate substrate kit (Bio-Rad, USA).

Cytokines and Nitric oxide (NO) measurements in RAW 264.7 cells

RAW 264.7 cells (a murine macrophage cell line; 1×10^6 cells/ml) were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), penicillin (50 µg/ml), and streptomycin (50 µg/ml) at 37 °C under 5 % CO₂ for 8 h in 12 well plates.

The activity of LPS contamination in recombinant proteins was inhibited by incubation with polymyxin B (10 µg/ml) for 30 min before stimulation. *E. coli* LPS (1 µg/ml, Sigma, USA) and DPBS (Gibco, USA) were used as positive and negative controls, respectively. Culture supernatants were collected at indicated times after stimulation. Amounts of cytokines (TNF-α, IL-6, IL-1β, IL-12p70 and IFN-γ) were measured using an enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (eBioscience Inc., USA). NO production was determined by measuring nitrite accumulation using the Griess reaction, as previously described [Im *et al.*, 2016b].

Cytokine measurements in splenocytes of naïve mice

To assay IFN-γ, IL-2, IL-4 and IL-5 levels, splenocytes (1 x 10⁶ cells/ml) were isolated from healthy 5-week-old BALB/c female mice (Orient-Bio, Korea), and stimulated with 10 µg/ml of rMdh, rTsf, or rRocF. After 24 h, amounts of IFN-γ, IL-2, IL-4 and IL-5 in culture supernatants were measured by ELISA according to the manufacturer's instructions (eBioscience Inc., USA). *E. coli* LPS (1 µg/ml, Sigma, USA) and media were used as positive and negative controls, respectively. Elimination of LPS from *B. abortus* antigens was done by pretreatment with polymyxin B. Animal care and handling were performed after obtaining approval from the Seoul National University Institutional Animal Care and Use Committee (IACUC; approval number SNU-150302-2-1).

Numbers of IgG, IFN- γ and IL-4 secreting cells among mouse splenocytes

Five 6-week-old BALB/c female mice were prepared per each group for three *B. abortus* antigens and control. Three *B. abortus* antigens emulsified with complete Freund's adjuvant at 1st injection and with incomplete Freund's adjuvant at 2nd injection. Mice were immunized with 30 μ g of purified *B. abortus* antigens at each injection with 2 weeks interval. After then, splenocytes of mouse were collected at day 28 after 1st injection. Levels of *B. abortus* antigens, rMdh, rTsf and rRocF, specific IgG memory B cells and IFN- γ and IL-4 secreting T-cells from spleen of immunized mice were measured using the Enzyme-Linked ImmunoSpot assay kit (ELISpot Basic kit; Mabtech AB, Sweden). For IgG memory B cells, 200 μ l of Mdh, Tsf, and RocF in PBS (50 μ g/ml) was added to the ELISpot plates after pretreatment with 70 % ethanol and incubated overnight at 4 °C. For IFN- γ and IL-4 secreting T cells, 200 μ l of monoclonal antibody against IFN- γ (AN18, 15 μ g/ml) and IL-4 (11B11, 15 μ g/ml) were added to the plates and coated by overnight incubation at 4 °C. After incubation, plates were extensively washed with PBS five times and blocked with RPMI1640 containing 10 % FBS for 30 min at RT. Upon removal of the medium, splenocytes were isolated from mice at 28 days after first immunization with *B. abortus* antigens and added to wells at 2×10^5 cells/well. Plates were incubated at 37 °C under 5 % CO₂ for 24 h for IgG or 48 h for IFN- γ or IL-4. After removing the cells, 100 μ l of biotinylated anti-IgG specific to IL-4 or IFN- γ antibodies in PBS containing 0.5 % FBS (PBS-0.5 % FBS) were added to each well. After incubation for 2 h at RT, plates were washed and streptavidin-HRP in PBS-

0.5 % FBS was added and incubated for 1 h at RT. Wells were then washed with PBS and antigen secreting cells (ASCs) were visualized upon adding ready-to-use TMB substrate solution. The number of ASCs were counted using an Eli.Scan+ (A.EL.VIS, Germany). The experiment was performed in triplicate.

Production of immunoglobulins in mice immunized with recombinant proteins

Five 6-week-old BALB/c female mice per control and treatment groups were prepared each time points (3, 7, 14 and 28 days). Each group was intraperitoneally immunized with 30 µg of purified *B. abortus* antigens emulsified with complete Freund's Adjuvant (CFA, Sigma, USA) on day 0 and with incomplete Freund's Adjuvant (IFA, Sigma, USA) on day 14. Serum samples were obtained for the detection of antibody response at 3, 7, 14 and 28 days after first immunization. Antibody production was measured by ELISA. In brief, 96-well microplates (Greiner Bio One, Germany) were coated by incubating the purified *B. abortus* antigens, rMdh, rTsf, or rRocF (5 ng per well), in coating buffer (14.2 mM Na₂CO₃, 34.9 mM NaHCO₃, 3.1 mM NaN₃, and pH 9.6) overnight at 4 °C. The plates were then blocked with 1 % bovine serum albumin (Sigma, USA) in PBS containing 0.1 % Triton X-100 (PBST) for 2 h at 37 °C. After washing with PBST, 1/200 diluted serum samples were reacted with coating antigens for 1 h at 37 °C. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:2,000, Bio-Rad, USA), IgG1 (1:2,000, Southernbiotech, USA), IgG2a (1:2,000, Southernbiotech, USA), and IgM (1:3,000, Jackson Immuno, USA) diluted in PBS containing 1 % BSA were used to detect IgG,

IgG1, IgG2a, and IgM, respectively. The color development was conducted using a 3,3',5,5'-tetramethyl-benzidine (TMB) substrate (Sigma, USA). The absorbance was measured using an automatic microplate reader (Molecular Device Co., USA) at 450 nm. The experiment was performed in triplicate.

Statistical Analysis

Statistical significance (P -value) was determined using the Student's t test using SPSS version 4.0 (Statistical Package for Social Science; MS, USA). Statistical significance was accepted for P values < 0.05 . Each experiment was performed in triplicate.

Results

Purification of recombinant rMdh, rTsf, and rRocF from *Brucella abortus*

Cloning of the genes encoding was performed as previously described [Sung *et al.*, 2014; Im *et al.*, 2016b]. These three genes encode for malate dehydrogenase, elongation factor, and arginase, respectively, in the pCold TF system led to expression of TF fusion proteins, which were purified using a histidine column. SDS-PAGE profiles indicated purified rMdh, rTsf, and rRocF were 85.71 kDa, 83.49 kDa, and 85.19 kDa sized, respectively. The recombinant proteins expressed with pCold TF vector were confirmed by Western blot using anti-histidine antibody

(Figure 17).

Productions of cytokines and NO in RAW 264.7 cells

Amounts of TNF- α in RAW 264.7 cells stimulated with rMdh, rTsf, or rRocF were higher than in those stimulated with DPBS at 4 or 8 h. However, stimulation with rMdh and rRocF were not increased at 24 h post stimulation while the stimulation of rTsf group was constantly increased by time dependent manner (* $P < 0.05$, ** $P < 0.01$) (Figure 18). Production of IL-6, IL-1 β , IL-12p70, IFN- γ and NO were not detected in the culture supernatants of RAW 264.7 cells stimulated with either of the three *B. abortus* antigens. Thus, *B. abortus* antigens induced only the production of TNF- α in RAW 264.7 cells.

Production of IFN- γ , IL-2, IL-4 and IL-5 in naïve mouse splenocytes

Amounts of the Th1-related cytokines IFN- γ and IL-2, and those of the Th2-related cytokines IL-4 and IL-5, in culture supernatants of the naïve mouse splenocytes were measured at 12 and 24 h after stimulation with rMsh, rTsf, or rRocF by ELISA. Productions of IFN- γ by mouse splenocytes stimulated with rMdh, rTsf, or rRocF were higher than those produced by non-treated controls ($P < 0.01$) (Figure 19A). High levels of IL-2 production were observed at 24 h after treatment with rMdh or rTsf ($P < 0.01$) (Figure 19B). However, the Th2-related cytokines, IL-4 and IL-5, were not detected in the culture supernatants of the splenocytes after the stimulation.

Analysis of antigen-specific Ig and cytokine secreting cells in the splenocytes of mice immunized with rMdh, rTsf, or rRocF

Numbers of IgG secreting B cells among mouse splenocytes at 28 days after immunization with *B. abortus* antigens were analyzed by ELISpot. A significant increase in these cells were only observed in the rMdh stimulated group (Figure 20A). Numbers of IFN- γ and IL-4 secreting T cells were also analyzed. Numbers of IFN- γ secreting T cells were significantly increased in rMdh and rTsf immunized cells ($P < 0.05$) (Figure 20B) while numbers of IL-4 secreting cells were increased by immunization group with rMdh, rTsf, or rRocF ($P < 0.05$) (Figure 20C).

Production of antibodies in mice immunized with rMdh, rTsf, or rRocF

Productions of IgG, IgG1 and IgG2a, and IgM were analyzed after immunizing mice with either of the three *B. abortus* antigens. IgM production against rMdh was significantly increased until day 14 post-immunization versus treatment naïve controls, and subsequently decreased. IgM production after immunization with rMdh, rTsf, or rRocF was higher than by non-treated group at day 7 ($P < 0.05$) (Figure 21A). IgG production was significantly increased by rMdh, rTsf, or rRocF from 7 days and plateaued at 28 days ($P < 0.05$) (Figure 21B). The production of IgG2a antibody was significantly elevated after immunization with rMdh or rTsf from 7 and 14 days after immunization, respectively. Antibody production peaked at 28 days in all experimental groups (Figure 21C). IgG1 antibody production exhibited the same

pattern as IgG2a antibody production (Figure 21D), although IgG1 antibody production was greater and commenced earlier than IgG2a antibody production after immunization with rMdh.

Discussion

Brucellosis is a widespread, contagious disease that affects a variety of domestic animals, in which it causes abortion and sterility. Diagnosis of the disease has been done based on microbiological, serological, or molecular methods [Han *et al.*, 2014]. Recently, researchers have focused on finding diagnostic antigens that do not exhibit cross-reactivity. Although several immune-reactive proteins have been suggested by these researchers, most of the proteins identified showed some level of cross-reactivity. This lack of specificity might be due to poor understanding of the immune mechanisms associated with *Brucella* infections. In the present study, three immunodominant proteins, as identified by Western-blot analysis of the sera of *B. abortus* 2308-challenged cattle [Lee *et al.*, 2015], were selected to have potential possibility for induction of immune responses after stimulation in the host and their *in vitro* and *in vivo* immunological responses were investigated.

Malate dehydrogenase (Mdh) is a multimeric enzyme that plays a crucial role in the tricarboxylic acid cycle of prokaryotic and eukaryotic cells. *B. abortus* Mdh is

identified by *in vivo*-induced antigen technology (IVIAT), and the vaccination with bacterially expressed *B. abortus* Mdh enhances protection against *Brucella* infection in elk [Lowry *et al.*, 2010]. Also, Mdh in *B. abortus* has been reported to be immunogenic and to be related to bacterial pathogenesis, especially colonization [Han *et al.*, 2010]. In addition, purified recombinant Mdh expressed in pMAL vector has been demonstrated to be a potential specific antigen for the serological diagnosis and early detection of bovine brucellosis [Reyes *et al.*, 2016]. Partial protective immunity to Mdh was also demonstrated in a challenge test using mice immunized with *Toxoplasma gondii* Mdh [Liu2016]. In present study, recombinant Mdh of *B. abortus* induced only TNF- α in RAW 264.7 cells and IFN- γ and IL-2 in naïve mice splenocytes. The patterns of TNF- α , IL-6 and NO production in RAW 264.7 cells in this study were different with those from our previous study with other recombinant proteins [Im *et al.*, 2016b], whereas IFN- γ and IL-2 production patterns were similar to those observed in our previous study [Im *et al.*, 2016b]. Numbers of antigen-secreting cells (ASCs) among the splenocytes of mice immunized with the three recombinant proteins were also similar to those observed previously with the exception of lower number of cells compared with r0628 and rTbpA [Im *et al.*, 2016b]. Therefore, it was confirmed that the immunogenicity of the recombinant protein Mdh increased the production of cytokines associated with immune responses.

Translation elongation factor EF-Tu is an essential component of the translational machinery, and may play additional roles. It was recently demonstrated EF-Tu is

released/secreted from bacterial cells and that it can modulate pathogen-host interactions. However, the mechanism responsible for disruption of immune regulation in host cells has not been determined [Nieves *et al.*, 2010; Sharma *et al.*, 2011]. Furthermore, recombinant translation elongation factor of *B. abortus* induced the production of TNF- α in RAW 264.7 cells up to 12 h after stimulation while the productions of IFN- γ and IL-2 were increased for up to 24 h in naïve mouse splenocytes. This phenomenon was different with *B. abortus* other cellular proteins [Im *et al.*, 2016b]. In this study, recombinant rTsf of *B. abortus* showed high production of cytokines and antigen-secreting cells with *in vitro* and *in vivo* experiments after stimulation.

Arginase is a metalloenzyme that hydrolyzes L-arginine to ornithine and urea, and its two isoforms exhibit differential subcellular localizations and tissue distributions. Arginase I is a cytosolic enzyme that is predominantly expressed in hepatocytes, whereas arginase II is a mitochondrial enzyme and is expressed in brain, kidneys, small intestine, monocytes and macrophages [Gogoi *et al.*, 2016]. Arginine metabolism is crucial for M1 and M2 polarization effects. M1 macrophages are proinflammatory in nature, produce NO and NO-derived peroxynitrite, and induce Th1 adaptive immune response [Gogoi *et al.*, 2016]. M2 macrophage differentiation is induced by elevated arginase levels and gives rise to anti-inflammatory and anti-parasitic Th2 responses [Gogoi *et al.*, 2016].

Although RocF has been identified as an immunoreactive protein by Western blot with sera from *B. abortus* 2380-challenged cattle, the role of RocF in immune

response has not been determined [Lee *et al.*, 2015]. In the present study, rRocF induced the productions of TNF- α and IFN- γ and the production of IgG in mice immunized with *B. abortus* antigens. Also, numbers of IL-4 secreting cells were increased among splenocytes of mice 28 days after immunization with *B. abortus* antigens. These results indicate RocF could be related with the induction of Th2 types of immune response. Collectively, our study suggests that these three cellular proteins of *B. abortus* might induce strong Th2-type related immune responses *in vivo*. Moreover, three *B. abortus* antigens could be a candidate biomarker for studying pathogenesis of *Brucella* infection or developing vaccine of brucellosis.

Table 4. Primer sequences used for the cloning *Brucella abortus* genes

Gene	Primer sequence (5' - 3')	Annealing temperature (°C)	Product size (bp)	Reference
Malate dehydrogenase (Mdh)	F: AATTCGGATCCATGGCACGCAACAAGATT R: AGGCGTCGACTTATTTTCAGCGACGGAGC	63	963	[Im <i>et al.</i> , 2017]
Elongation factor (Tsf)	F: AATTCGAATTCATGAGCATTTCGCGCATCT R: AGGCCTGCAGTCAGCCCTTGGCGGCTGCGGC	63	918	[Im <i>et al.</i> , 2017]
Arginase (RocF)	F: AGCGCGGATCCATGCATTGTAAGATTCTG R: AGGCCTGCAGTCAATAGCTGATGGTCGG	63	918	[Im <i>et al.</i> , 2017]

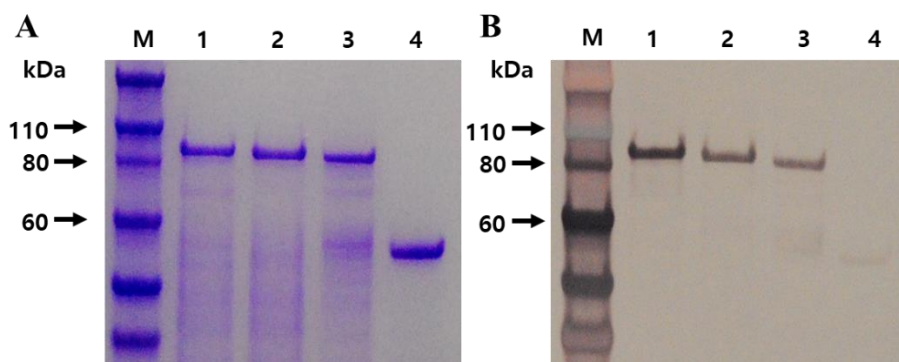


Figure 17. Analysis of purified recombinant Mdh, Tsf, and RocF of *Brucella abortus*. SDS-PAGE (A) and Western blot (B) analysis of soluble protein fractions of pCold TF expressed with expected size. M: molecular weight markers, Lane 1: rMdh (85.71 kDa), Lane 2: rTsf (83.49 kDa), Lane3: rRocF (85.19 kDa), Lane 4: vehicle (52 kDa).

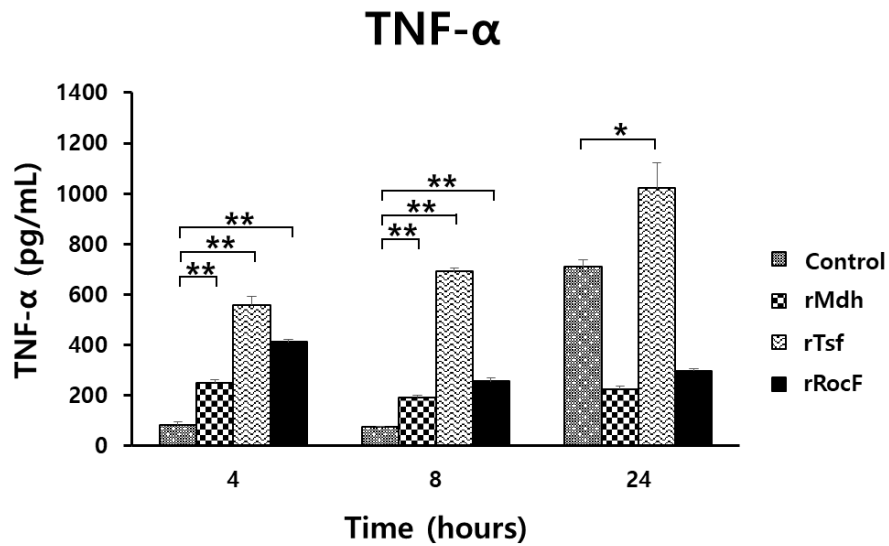


Figure 18. Productions of TNF- α in RAW 264.7 cells stimulated with *B. abortus* antigens. Only rTsf increased the production of TNF- α until 24 h after stimulation, whereas rMdh and rRocF increased the production of TNF- α at 4 and 8 h versus nontreated controls. (* $P < 0.05$, ** $P < 0.01$).

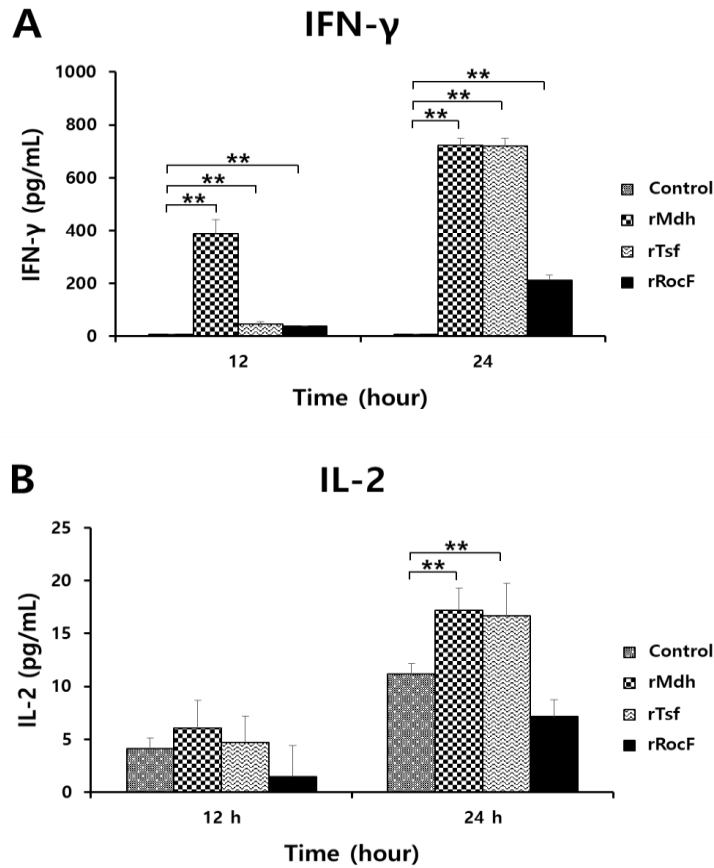


Figure 19. Productions of IFN- γ and IL-2 by naïve mouse splenocytes. (A) Production of IFN- γ was increased at 12 and 24 h after stimulating cells with purified rMdh, rTsf, or rRocF. (B) Production of IL-2 was increased at 24 h after stimulating cells with purified rMdh or rTsf. The production of IFN- γ was induced in splenocytes after stimulation with all three *B. abortus* antigens groups compared to the control group. The production of IL-2 in splenocytes was induced in the two experimental groups stimulated with rMdh and rTsf compared to control group. (** $P < 0.01$).

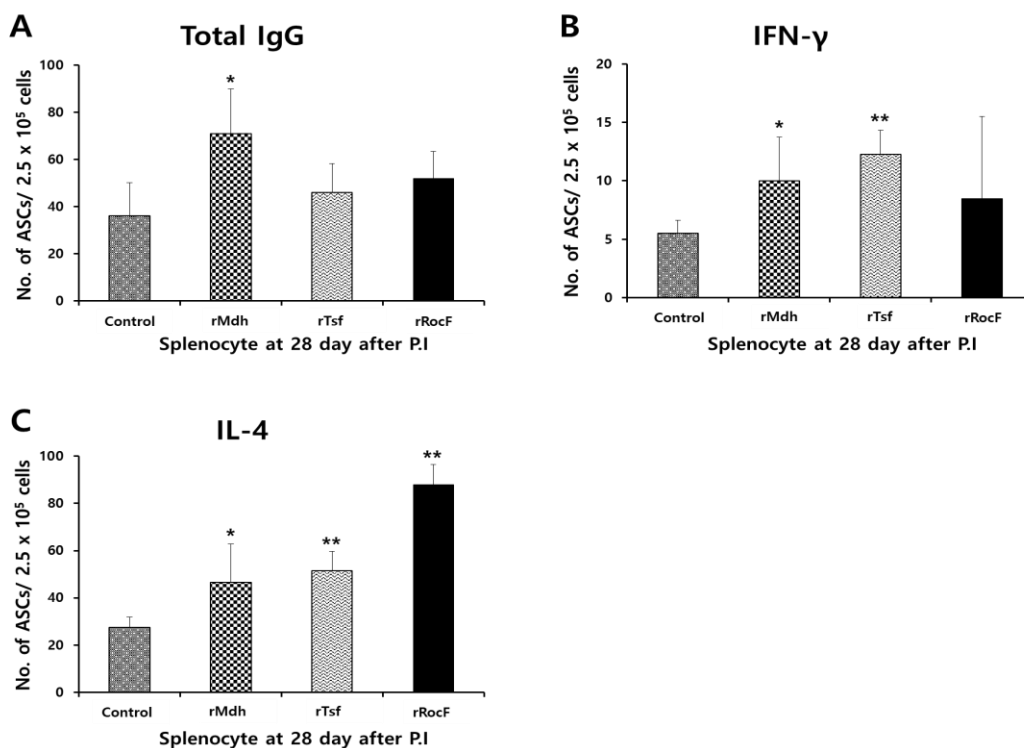


Figure 20. Numbers of antigen-secreting cells (ASCs) in the splenocytes of mice immunized with rMdh, rTsf, or rRocF of *Brucella abortus*. (A) Total IgG production was increased by rMdh at 28 days post-immunization. (B) Production of IFN-γ was at 28 days post-immunization with rMdh or rTsf. (C) Production of IL-4 was increased at 28 days post-immunization by rMdh, rTsf, or rRocF. Three *B. abortus* antigens induced different number of antigen-secreting cells after the stimulation. Of the cellular antigens of *B. abortus*, rMdh showed higher antigenicity compared to other *B. abortus* antigens. (* $P < 0.05$, ** $P < 0.01$).

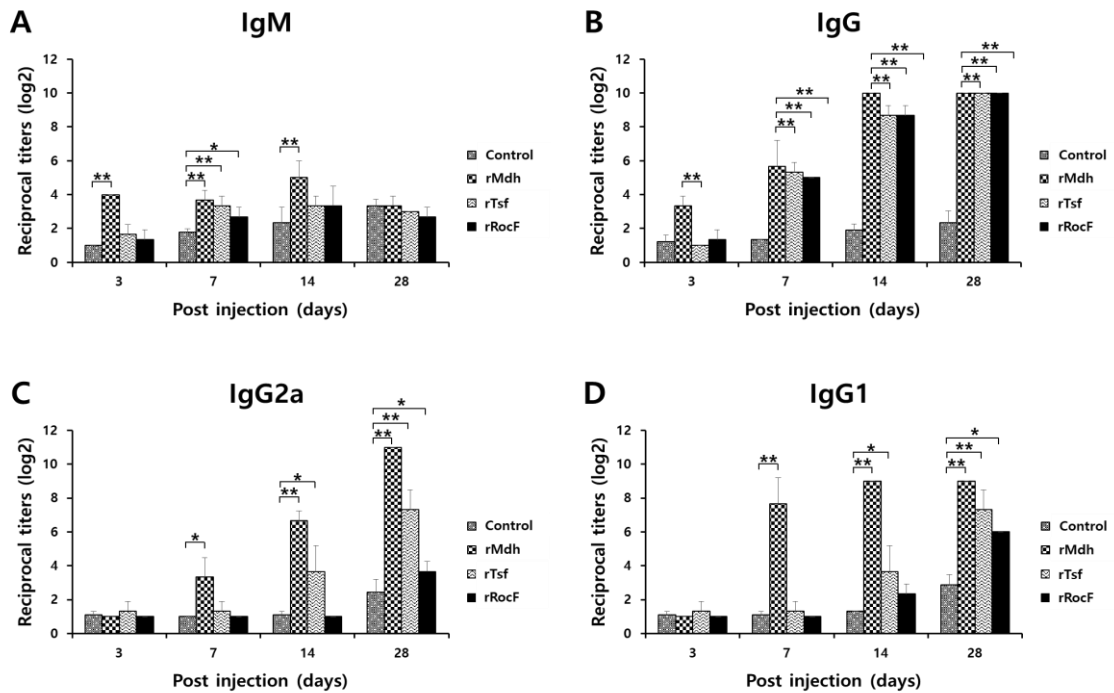


Figure 21. Humoral immune responses (IgM, IgG, IgG2a and IgG1) in mice immunized with rMdh, rTsf, or rRocF. (A) IgM production was increased at 7 days post-immunization with rMdh, rTsf and rRocF. rMdh had the greatest effect on IgM production at 14 days post-immunization. (B) IgG production was time-dependently increased by immunization with rMdh, rTsf and rRocF. Productions of IgG2a (C) and of IgG1 (D) were induced 7, 14, and 28 days after immunization with rMdh, rTsf, or rRocF, respectively. Therefore, all three *B. abortus* antigens, rMdh, rTsf, rRocF, showed the production of the antibodies by time-dependent. Especially, IgG, IgG2a and IgG1 productions were highly increased at 28 day while the production of IgM was increased at early time after stimulation with three *B. abortus* antigens. (* $P < 0.05$, ** $P < 0.01$).

Chapter III

Comparative Analysis of Immune Responses in *in vitro* and *in vivo* after Stimulation of *Brucella abortus* Outer Membrane Antigens, OMP10, OMP19 and OMP28

Abstract

Brucella infection is accompanied by cytokine production, which serves as an important factor to evaluate the innate and adaptive immune responses. Several researchers have been investigating the mechanisms involved in *Brucella* infection in the host. Here, we conducted an analytical study to define pathogenic pathways and immune mechanisms involved in *Brucella* infection by investigating the antigenic efficacy of recombinant outer membrane protein 10 (rOMP10), outer membrane protein 19 (rOMP19), and outer membrane protein 28 (rOMP28) *in vitro* and *in vivo* upon stimulation/immunization. Cytokine production was analyzed by nitric oxide (NO) assay and enzyme-linked immunosorbent assay (ELISA) after stimulation of RAW 264.7 cells and naive splenocytes with *B. abortus* antigens. Our

results show that levels of NO, tumor necrosis factor (TNF)- α , and interleukin (IL)-6 increased in RAW 264.7 cells in a time-dependent manner following *B. abortus* antigens stimulation. In contrast, levels of interferon (IFN)- γ and IL-2 increased in naive splenocytes after stimulation with *B. abortus* antigens. ELISA and ELISpot assays were performed after immunization of mice with *B. abortus* antigens. rOMP28 greatly increased IFN- γ , IL-2, and TNF- α levels than IL-4 and IL-6 levels in vitro. Of the *B. abortus* antigens, rOMP19 elicited a mixed Th1/Th2 immune response by increasing the number of IgG-secreting cells *in vivo*.

Keywords: *Brucella abortus*, Cytokine, Immunogenicity, Outer membrane protein, *B. abortus* antigens.

Introduction

Brucella is a Gram-negative bacterium and causative agent of a serious zoonotic disease, brucellosis. The genus *Brucella* has been classified according to its primary preferred host and antigenic variations [Ko *et al.*, 2003; Foster *et al.* 2007; Scholz *et al.*, 2010; Whatmore *et al.*, 2014]. *Brucella* infections induce a variety of clinical symptoms in both animals and humans [Franco *et al.*, 2007].

Brucella spp. are able to easily establish chronic infections in the host cell by avoiding lysosomal degradation and maintaining proliferation within macrophages [Detilleux *et al.*, 1990a; Detilleux *et al.*, 1990b]. Lipopolysaccharide (LPS) is the most important virulence factor that activates the innate immune system; *Brucella* strains are deficient in LPS and, hence, have lesser virulence and stronger ability to survive. However, LPS-based serological tests may give false-positive results, owing to the similarity between *Brucella* and gram-negative bacteria, such as *Yersinia enterocolitica* O:9 [Kittelberger *et al.*, 1998], *Escherichia coli* [Oñate *et al.*, 1999], and *Salmonella urbana* [Nielsen *et al.*, 2007]. Therefore, several studies have aimed to investigate other factors, such as outer membrane proteins (OMPs) and their potential applications as diagnostic markers and vaccines against brucellosis [Sowa *et al.*, 1991; Tibor *et al.*, 1996; Kovach *et al.*, 1997]. Recent studies have focused on various OMPs, such as OMP28, OMP2b, OMP10, OMP16, and OMP19 [Tibor *et al.*, 1999; Sung *et al.*, 2014; Simborio *et al.*, 2015]. Of these, OMP28 displays significant potential as a diagnostic candidate that may trigger protective effects *in*

vivo and show high efficiency in serological tests against brucellosis [Lindler *et al.*, 1996; Lim *et al.*, 2012a; Lim *et al.*, 2012b; Tiwari *et al.*, 2013]. Attempts have been made to clone these OMPs and other proteins to investigate if their immunogenicities may allow LPS-free diagnosis following infection with *Brucella* spp.

Immune responses in the host are caused following pathogenic infection, which triggers the production of proinflammatory cytokines, such as interleukin (IL)-1 β , IL-4, IL-6, IL-12, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α . The production of these cytokines results in the disturbance of immune responses in the host, as these molecules participate in cellular and/or humoral immune responses [Gu *et al.*, 2013; Im *et al.*, 2016b]. Therefore, the analysis of the cytokine production may explain the correlation between the invasion and localized inflammation by *B. abortus* infection. Many researchers have reported Th1 (cellular) and Th2 (humoral) immune responses following infection [Barquero-Calvo *et al.*, 2013; Im *et al.*, 2016b]. Although several immunogenic antigens have been investigated, only a few immune mechanisms have been identified in brucellosis.

In this study, 3 *B. abortus* antigens, rOMP10, rOMP19, and rOMP28, were selected to study the pathogenic pathways and evaluate their immunogenicities in hosts through the investigation of immune responses elicited in *in vitro* and *in vivo* tests.

Materials and Methods

Construction of OMP10, OMP19, and OMP28 expression clones

Total genomic DNA was prepared from *Brucella abortus* 544 (ATCC23448) culture using a G-spin™ Genomic DNA Extraction kit for bacteria (Intron, Korea). Genes encoding OMP10, OMP19, and OMP28 of *B. abortus* were amplified by PCR with primers pairs: OMP10 sense 5'-AGCAGAATTCATGAAACGCTTCCGCA-3', OMP10 anti-sense 5'-ATTACTGCAGTCAGCCGGCGTTGC-3', OMP19 sense 5'-AGCAGGATCCATGGGAATTTCAAAAGCAAG-3', OMP19 anti-sense 5'-ATACTGCAGTCAGCGCGACAGCG-3', and OMP28 sense 5'-GATCGGATCCAACACTCGTGCTAGCAATTTT-3', OMP28 anti-sense 5'-GATCAAGCTTTTACTTGATTTCAAAAACGAC-3'. The amplified DNA were digested with appropriate restriction enzymes, then ligated into a pCold TF vector (Takara, Japan), in which the recombinant proteins were expressed fused to the TF agent.

Expression and purification of rOMP10, rOMP19, and rOMP28 proteins

The expression and purification of recombinant proteins were modified as previously described [Lim *et al.*, 2012a]. Briefly, *E. coli* containing the fusion plasmid were cultured by inoculating 20 ml of the bacteria into one liter of ampicillin containing LB broth and incubated at 37 °C for 7 h. Isopropyl β -D-1-thiogalactopyranoside (IPTG, Amresco, USA) was then added to a final concentration of 0.3 mM and further incubated at 37 °C for 2 h. Bacterial cells were

subsequently harvested by centrifugation at 4,400 x g for 20 min, after which the supernatant was discarded and resuspended in 40 ml of column buffer (20 mM Tris HCl, 8 M Urea, 500 mM NaCl, 20 mM Imidazole, 1 mM β -mercaptoethanol, pH 8.0). The samples were then sonicated at 10,000 Hz in an ice-water bath and centrifuged at 4,400 x g for 20 min to collect the supernatant. The supernatant was then loaded into a His SpinTrap (GE Healthcare, UK) column according to the manufacturer's instructions.

SDS-PAGE and Western blot

The purified recombinant proteins were diluted with sample buffer and boiled for 10 min at 100 °C. After electrophoresis, samples were visualized by staining with Coomassie Brilliant Blue R-250 (Intron, Korea). Proteins resolved by SDS-PAGE were transferred to nitrocellulose membrane (Invitrogen, USA) for 25 minutes using an iBlot® transfer device (Invitrogen, USA). Next, membranes were blocked by incubation in 5 % skim milk (BD, USA) for 1 h at room temperature (RT), washed three times with TBS, and then incubated with anti-histidine (1:2,000 dilution, AprilBio Co., Ltd, Korea) for 3 h at RT. The membranes were washed again three times for 10 min, after which they were incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (1:2,000 dilution, Bethyl, USA,) for 1 h at room temperature. After the final wash, proteins were visualized with an AP conjugate substrate kit (Bio-Rad, USA).

Production of nitric oxide (NO) and cytokines in RAW 264.7 cells

After incubating RAW 264.7 cells for 8 h in 12 well plates containing 1×10^6 cells/ml, they were stimulated with 10 $\mu\text{g/ml}$ rOMP10, rOMP19, and rOMP28. Contamination by LPS in *B. abortus* antigens was inhibited by incubation with polymyxin B (10 $\mu\text{g/ml}$) for 30 min before stimulation of macrophages. The culture supernatants were collected 4, 8 and 24 h after stimulation. The amounts of TNF- α , IL-6, IL-1 β , IL-12p70, and IFN- γ were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (eBioscience inc., USA). The level of NO was determined by measuring the nitrite accumulation based on Griess reagent system.

Production of cytokines in naïve splenocytes of mice

For the IFN- γ , IL-2, IL-4 and IL-5 assays, splenocytes (1×10^6 cells/ml) were isolated from healthy female BALB/c mice that were 5-weeks of age (Orient-Bio, Korea) and then stimulated with 10 $\mu\text{g/ml}$ of rOMP10, rOMP19, and rOMP28. After 24 h, the amounts of IFN- γ , IL-2, IL-4 and IL-5 in the culture supernatants were measured by ELISA according to the manufacturer's instructions (eBioscience Inc., USA). LPS (Sigma, USA) and media were used as positive and negative controls, respectively. All stimuli were pretreated with polymyxin B to avoid the activity of LPS contamination. All care and handling of animals was performed with the approval of the Seoul National University Institutional Animal Care and Use Committee (IACUC) and approval number was SNU-150302-2-1.

Production of immunoglobulins and antigen (OMPs)-specific antibodies in mice immunized with recombinant proteins.

We immunized 6-week-old BALB/c mice with an intraperitoneal injection of 30 µg purified *B. abortus* antigens, rOMP10, rOMP19, or rOMP28 mixed with complete Freund's Adjuvant (CFA, Sigma) on day 0 and incomplete Freund's Adjuvant (IFA, Sigma) on day 14. Five mice were immunized with *B. abortus* antigens for each group. Sera for antibody response detection were obtained on 3, 7, 14, and 28 days after the first immunization. Briefly, 96-well microplates were coated by incubating purified *B. abortus* antigens (5 ng/well) in a coating buffer (14.2 mM sodium carbonate [Na₂CO₃], 34.9 mM sodium bicarbonate [NaHCO₃], and 3.1 mM sodium azide [NaN₃], pH 9.6) overnight at 4 °C for ELISA. The plates were blocked with 1 % bovine serum albumin (BSA; Sigma) in a solution of phosphate-buffered saline (PBS) containing 0.1 % Triton X-100 (PBST) for 2 h at 37 °C. After washing with PBST, 1/200 diluted serum sample was added to the wells and incubated for 1 h at 37 °C. Horseradish peroxidase (HRP)-conjugated goat antimouse IgG, IgG1, IgG2a (1:2,000, Bio-Rad), and IgM (1:3,000, Jackson Immuno Research, West Grove, PA, USA) diluted in PBS containing 1 % BSA were used to detect IgG, IgG1, IgG2a, and IgM, respectively. Color developed when the substrate, 3,3',5,5'-tetramethyl-benzidine (TMB) (Sigma), was added. The absorbance was measured at 450 nm using an automatic microplate reader (Molecular Device Co., Silicon Valley, CA, USA). The levels of antibodies specific

to rOMP10, rOMP19, and rOMP28 were measured after incubation of sera from mice immunized with *B. abortus* antigens at 37 °C for 1 h. For the detection of antibodies specific to *B. abortus* antigens, 96-well microplates were coated with purified *B. abortus* antigens (25 ng/well) in the coating buffer overnight at 4 °C. The plates were blocked with 1 % BSA in PBST for 2 h at 37 °C. After washing with PBST, 1/160 diluted serum sample was added to the wells and incubated for 1 h at 37 °C. HRP-conjugated goat anti-mouse IgG (H+L) (1:2,000, Bio-Rad) diluted in PBS containing 1 % BSA was used to detect IgG. Color developed when the substrate, TMB, was added, and the absorbance was measured at 450 nm using an automatic microplate reader.

Measurement of cells secreting IgG, IFN- γ , and IL-4 in mouse splenocytes

Recombinant rOMP10-, rOMP19-, and rOMP28-specific IgG memory B cells and T cells secreting IFN- γ and IL-4 from spleens of immunized mice were measured using an enzyme-linked immunospot (ELISpot Basic) assay kit according to the manufacturer's instructions (Mabtech AB, Stockholm, Sweden). The number of cells secreting IgG, IFN- γ , and IL-4 were counted using Eli.Scan+ (A.EL.VIS, Hannover, Germany).

Statistical analysis

Statistical significance was calculated with Student's *t*-test using the Statistical Package for Social Science (SPSS) software version 4.0 (MS, Chicago, IL, USA).

Differences were considered to be significant if a value of $P < 0.05$ was obtained.

All experiments were repeated at least 3 times

Results

Cloning and purification of recombinant OMP10, OMP19, and OMP28 of *B. abortus*

Cloning of genes encoding OMP10, OMP19, and OMP28 was performed as described in a previous study [Simborio *et al.*, 2015] with some modifications. SDS-PAGE profiles revealed the size of purified rOMP10, rOMP19, and rOMP28 to be approximately 65.86, 71, and 80 kDa, respectively (Figure 22A). Western blot analysis was performed to evaluate the cellular antigens of *B. abortus* after protein purification, and the expected sizes of rOMP10, rOMP19 and rOMP28 were determined (Figure 22B).

Production of NO and inflammatory cytokines in RAW 264.7 cells

We failed to observe any increase in NO production in groups stimulated with rOMP10 and rOMP28 at 24 h. On the other hand, cells stimulated with rOMP19 showed an increase in NO production at 24 h as compared to the control group ($P < 0.01$) (Figure 23A). The level of TNF- α detected in groups stimulated with all 3 *B. abortus* antigens was higher than that observed in the control group and these

changes occurred in a time-dependent manner (Figure 23B). The production of IL-6 increased after 8 h in response to stimulation with rOMP10 and rOMP19. In contrast, stimulation with rOMP28 induced the production of IL-6 at 24 h after stimulation (Figure 23C). We failed to detect IL-1 β , IL-12 and IFN- γ in cells stimulated with *B. abortus* antigens. Thus, *B. abortus* antigens showed immune-stimulating activities in RAW 264.7 cells, as evident from the production of TNF- α and IL-6.

Production of IFN- γ and IL-2 in naïve mouse splenocytes

The production of IFN- γ was higher in mouse splenocytes stimulated with rOMP28 as compared with the control group and differences were observed in a time-dependent manner ($P < 0.01$) (Figure 24A). In addition, the level of IL-2 increased in cells treated with rOMP28 as compared with the control group at 24 h ($P < 0.01$) (Figure 24B). Therefore, rOMP28 induced high immune-stimulating activities through the production of IFN- γ and IL-2 in naïve mouse splenocytes.

Analysis of antigen-secreting cells in splenocytes from mice immunized with rOMP10, rOMP19, and rOMP28

The number of antigen-specific IgG-secreting B cells from mice splenocytes was analyzed using ELISpot following 28 days of immunization with *B. abortus* antigens. The number of antigen-specific IgG secreting B cells significantly increased in mice immunized with rOMP19 compared that in mice in the nonimmunized group, while no increase was observed in response to rOMP10 and rOMP28. No difference was

reported in the number of IFN- γ - and IL-4-secreting T cells involved in the production of Th1-related and Th2-related cytokines, respectively, for the groups (Table 5).

Production of antibodies in mice immunized with rOMP10, rOMP19, and rOMP28

The production of antibodies specific to each recombinant protein was confirmed from the sera obtained in the *in vivo* immunoreactivity test (Figure 25). The *B. abortus* antigen-specific antibody was generated on day 7 after immunization (Figure 25A, B). However, the level of antibody to rOMP28 was found to be lower than that for other *B. abortus* antigens (Figure 25C). Nevertheless, this observation suggests that *B. abortus* antigens used in this study may induce protein-specific immune responses *in vivo*. The level of IgM antibody against rOMP28 was significantly increased compared with that in the control group on day 3, while the level of antibody to rOMP19 increased on day 14 ($P < 0.05$) (Figure 26A). The level of IgM produced in groups immunized with each of the 3 *B. abortus* antigens was similar to that observed in the control group on days 7 and 28, while the level of IgG in all 3 recombinant groups was significantly higher than that in the control group on day 14 after immunization ($P < 0.01$) (Figure 26B). Stimulation with rOMP19 led to an increase in the production of IgG1 after 3 days, whereas rOMP28 group showed an increase in IgG1 level after 14 days. The production of Th1-related antibody, IgG2a, gradually increased in rOMP19 group after 7 days, but only after

28 days of immunization in rOMP10 and rOMP28 groups. On day 28, all 3 groups showed an increase in the level of IgG2a compared with the control group (Figure 27A). The production of Th-2 related antibody, IgG1, in rOMP10 group was significantly increased on day 3, while that in rOMP19 group increased 7 days after immunization. All 3 groups showed an increase in IgG1 level on day 14 after immunization (Figure 27B).

Discussion

In this study, we cloned and expressed the 3 genes encoding immunogenic outer membrane proteins as *B. abortus* antigens using pCold TF expression system [Simborio *et al.*, 2015]. *B. abortus* antigens were identified with SDS-PAGE and western blot analysis at their expected sizes following purification using anti-histidine column. In addition, it was evaluated immunological characteristics of these cellular antigens from *B. abortus*.

Many studies have focused on the investigation of immune responses, including evaluation of the production of proinflammatory cytokines such as TNF- α , IL-6, IFN- γ , and IL-4 following stimulation and/or immunization with various antigens of *Brucella* spp. *in vitro* [Gu *et al.*, 2013; Sung *et al.*, 2014; Hop *et al.*, 2015]. Selected *B. abortus* antigens, rOMP10, rOMP19 and rOMP28, induced the production of TNF- α and IL-6 in RAW 264.7 cells after 4 and 8 h of stimulation. The protein

rOMP19 induced the production of exceptionally high levels of these cytokines than other *B. abortus* antigens, consistent with the results of other studies [Zhan *et al.*, 1993; Giambartolomei *et al.*, 2004; Sung *et al.*, 2014]. The production of NO significantly increased 24 h after stimulation of cells with rOMP19 as compared with control. The production of IFN- γ and IL-2 was high at 24 h after stimulation with rOMP28 in splenocytes from naive mice, whereas the stimulation with rOMP10 and rOMP19 induced the production of these cytokines at very low levels. IFN- γ is one of the major cytokines used to evaluate immune response both *in vitro* and *in vivo* after stimulation or immunization with *B. abortus* antigens. These results imply that rOMP28 triggered Th1 cell-mediated immune response via secretion of IFN- γ and IL-2. These findings are in line with the observation of high level of IFN- γ produced in splenocytes by other antigen sources without LPS [González-Smith *et al.*, 2006; Cha *et al.*, 2010; Sung *et al.*, 2014]. The production of antigen-specific IgM and IgG was observed in mice immunized with these 3 *B. abortus* antigens. The production of antigen-specific IgM was lower than that of antigen-specific IgG. All 3 *B. abortus* antigens showed strong antigen-specific IgG production 28 days after stimulation. In particular, rOMP19 showed the strongest immunogenicity from day 3 to 28 after immunization. Moreover, IgG1 and IgG2a production increased in a time-dependent manner, indicative of the shift of immunological response from Th1- to Th2-mediated response. The production of antigen-specific IgG-secreting B cells and IFN- γ - or IL-4-secreting T cells in the spleen was observed in mice immunized with rOMP10, rOMP19, and rOMP28. However, no difference was observed in levels of

IFN- γ - or IL-4-secreting T cells in mice immunized with the 3 *B. abortus* antigens as compared with the control group. Therefore, results of this study contribute to the understanding of the immunogenicity of rOMP10 and rOMP19. These OMPs have been generally studied to develop vaccines against diseases caused by various species, including *Anaplasma marginale* [Ducken *et al.*, 2015], *S. enterica* [Hamid *et al.*, 2008], *Haemophilus parasuis* [Li *et al.*, 2016], and *E. coli* [Montero *et al.*, 2014]. Pathogenic infections against host cells and the consequent cytokine production have been investigated thoroughly in the field of immunology and vaccine development [Eckmann *et al.*, 2001; Luo *et al.*, 2003; Boer *et al.*, 2015; Hicks *et al.*, 2015]. Thus, the evaluation of proinflammatory cytokine production is essential for the analysis of the protective immunity against pathogens. IFN- γ , IL-2, IL-4, IL-6 and TNF- α are some of the major target cytokines used to evaluate immunogenicity after stimulation and/or immunization with *B. abortus* antigens. In addition, IFN- γ and IL-2 are induced by spleen cells after immunization with live antigens [Zhan *et al.*, 1995]. IgG response is another factor involved in imparting protection against antigens. These results indicate that *B. abortus* antigens drive a shift from Th1 to Th2 immune responses after immunization of mice. Some studies have reported that Th1/Th2 balance is disturbed in response to stress or infection by antigens, resulting in the shift toward Th2-dominant immunity [Iwakabe *et al.*, 1998; Elenkov *et al.*, 2004]. Furthermore, the levels of IgG1 and IgG2a were shown to increase after immunization with the recombinant outer membrane protein 25 in mice, although no significant increase in cytokine-secreting splenocyte population

was observed [Goel *et al.*, 2012].

In conclusion, this study evaluated the immunogenicities of 3 cellular antigens (rOMP10, rOMP19 and rOMP28) of *B. abortus* by determining various immune responses. Of rOMPs, rOMP10 and rOMP19 showed strong immune responses *in vitro* and *in vivo*, although we failed to observe any significant increase in levels of IFN- γ and IL-2 in naive mice splenocytes. Thus, these proteins may serve as possible candidates for further investigation of the pathogenesis of *Brucella* infection.

Table 5. Number of antigen-secreting cells (ASC) from splenocytes of mice immunized with rOMP10, rOMP19, and rOMP28 of *Brucella abortus* at 28 days post immunization (** $P < 0.01$)

Immunized group	No. of IgG-secreting cells	No. of IFN- γ -secreting cells	No. of IL-4-secreting cells
PBS	36.17 \pm 13.96	5.5 \pm 1.11	27.5 \pm 4.28
rOMP10	28.5 \pm 7	3.75 \pm 5.19	14.5 \pm 3.87
rOMP19	210.75 \pm 35.42**	8.25 \pm 3.77	31.25 \pm 7.63
rOMP28	30 \pm 4.16	7.25 \pm 2.87	18.75 \pm 8.81

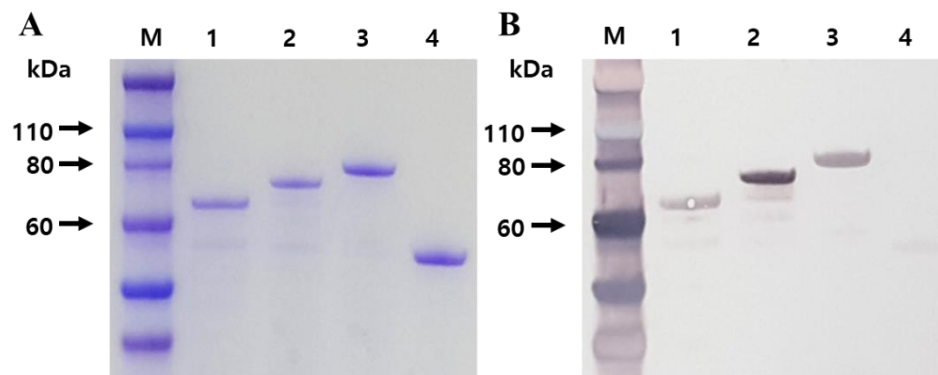


Figure 22. Analysis of purified recombinant OMP10, OMP19, and OMP28 of *B. abortus*. SDS-PAGE (A) and Western blot (B) analysis of soluble protein fractions of pCold TF expressed with expected size. M: molecular weight markers, Lane 1: outer membrane protein 10 (OMP10) (65.86 kDa), Lane 2: outer membrane protein 19 (OMP19) (71 kDa), Lane 3: outer membrane protein 28 (OMP28) (80 kDa), Lane 4: TF protein (52 kDa).

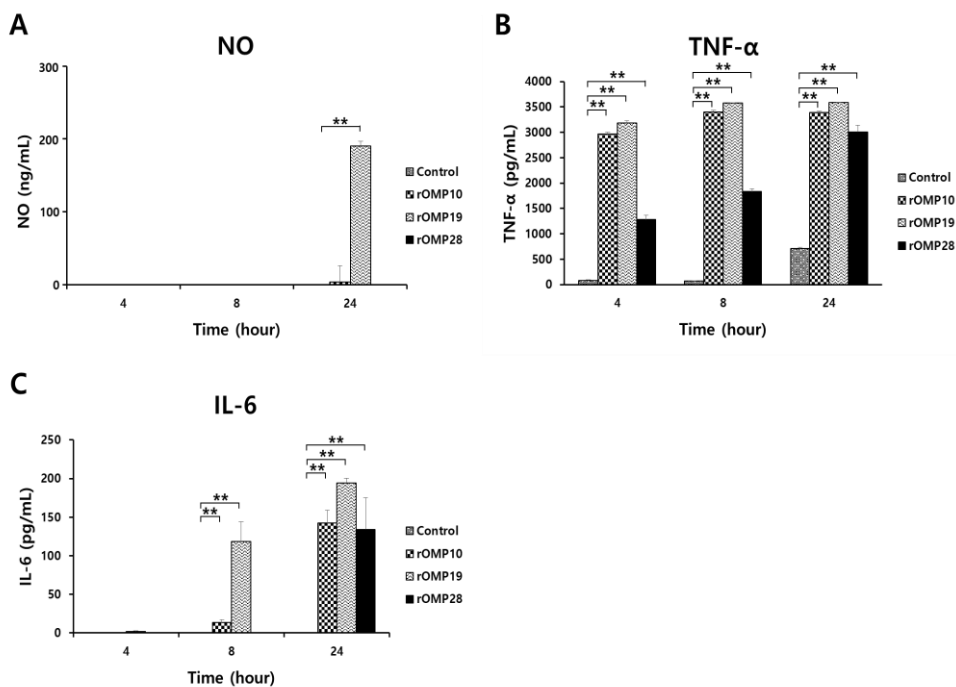


Figure 23. Production of nitric oxide (NO) and cytokines in RAW 264.7 cell after stimulation with *B. abortus* antigens. (A) Production of NO was increased after stimulation with rOMP19 at 24 h. (B) Production of TNF- α was significantly increased after stimulation with rOMP10, rOMP19, and rOMP28 in a time dependent manner. (C) Production of IL-6 was increased after 8 h of stimulation with rOMP10 and rOMP19, whereas stimulation with rOMP28 induced production of IL-6 at 24 h. (** $P < 0.01$).

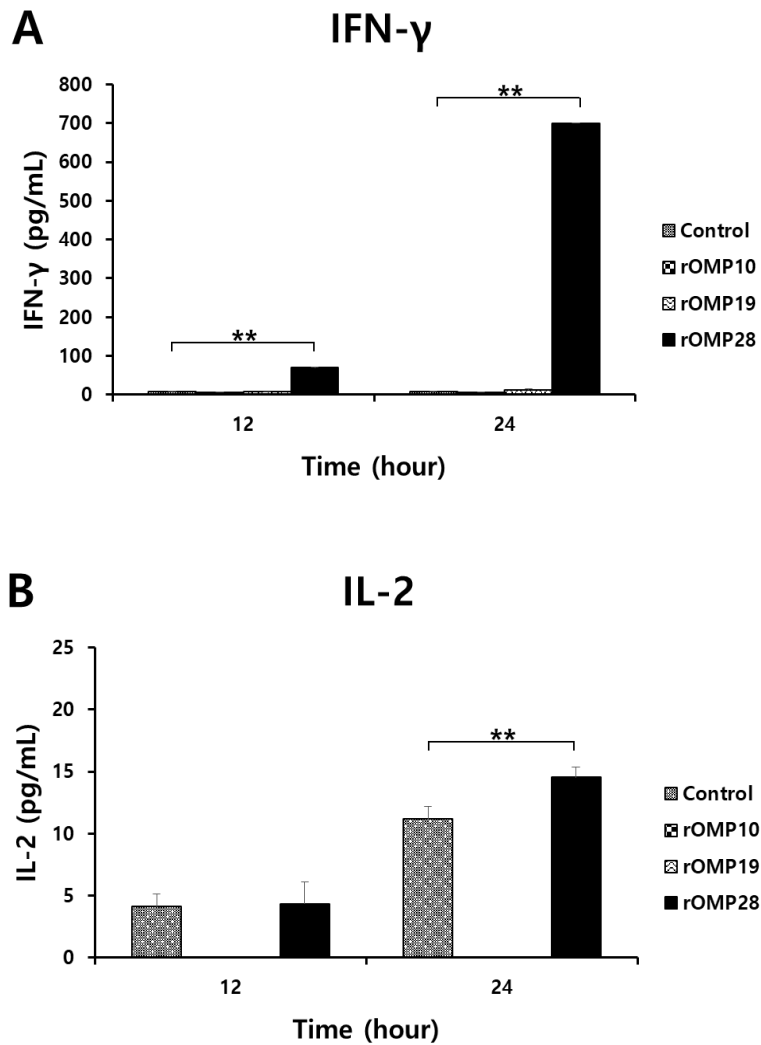


Figure 24. Production of IFN- γ and IL-2 by naïve mice splenocytes. (A) Production of IFN- γ was increased after stimulation with purified rOMP28 at 12 h and 24 h post stimulation. (B) Production of IL-2 was increased after stimulation with purified rOMP28 at 24 h post stimulation. (** $P < 0.01$).

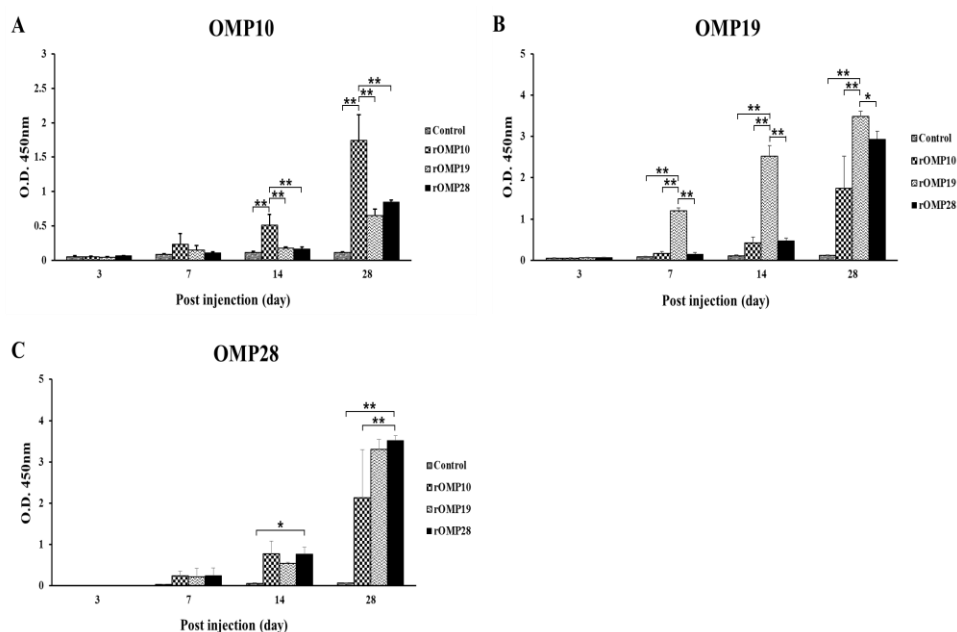


Figure 25. Detection of antigen-specific antibodies in immunized mice after reaction with recombinant TF protein. Antibodies to the rOMP10 and rOMP19 cellular antigens were increased at Day 14 after immunization except the rOMP28 immunized group. The production of antibodies the rOMP10 (A), rOMP19 (B) and rOMP28 (C) proteins were detected by ELISA using the sera from the 3 *B. abortus* antigens immunized mice groups. The antibodies to *B. abortus* antigens were produced time-dependent manner. At 28 days after the immunization, the production of all antibodies were increased in the 3 immunized mice groups (rOMP10, rOMP19 and rOMP28) compared to control mice group. (* $P < 0.05$, ** $P < 0.01$).

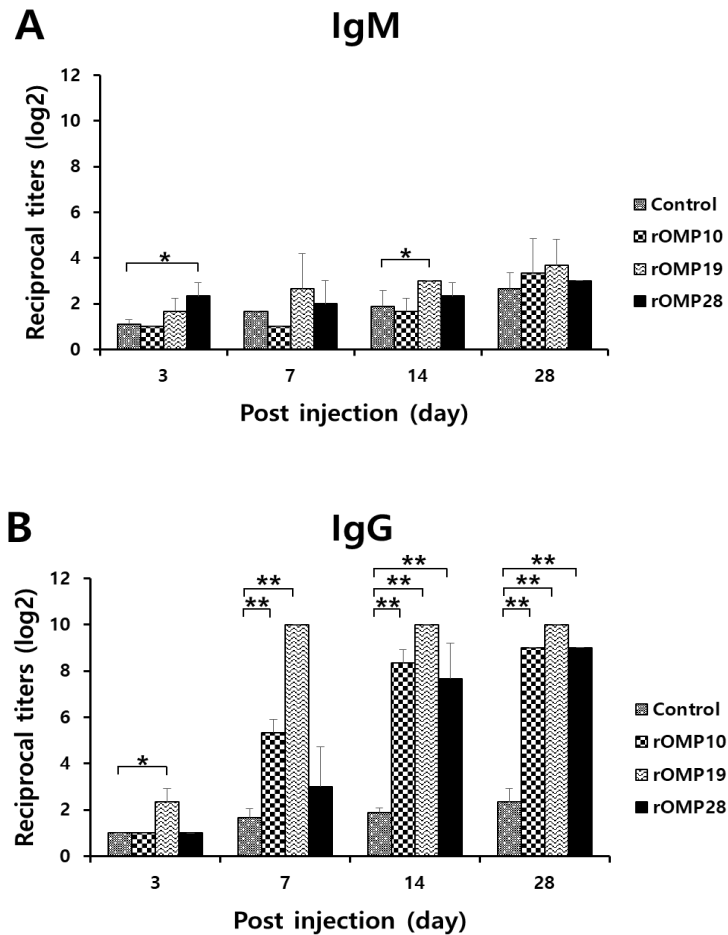


Figure 26. Humoral immune responses induced in mice by immunization with rOMP10, rOMP19 and rOMP28 of *B. abortus* (IgM and IgG). (A) Different levels of IgM production were observed after immunization with rOMP28 protein at 3 days and rOMP19 protein at 14 days. (B) Production of IgG was increased after immunization with rOMP10, rOMP19 and rOMP28 in a time dependent manner. Stimulation of rOMP19 increased from 3 days after immunization. (* $P < 0.05$, ** $P < 0.01$).

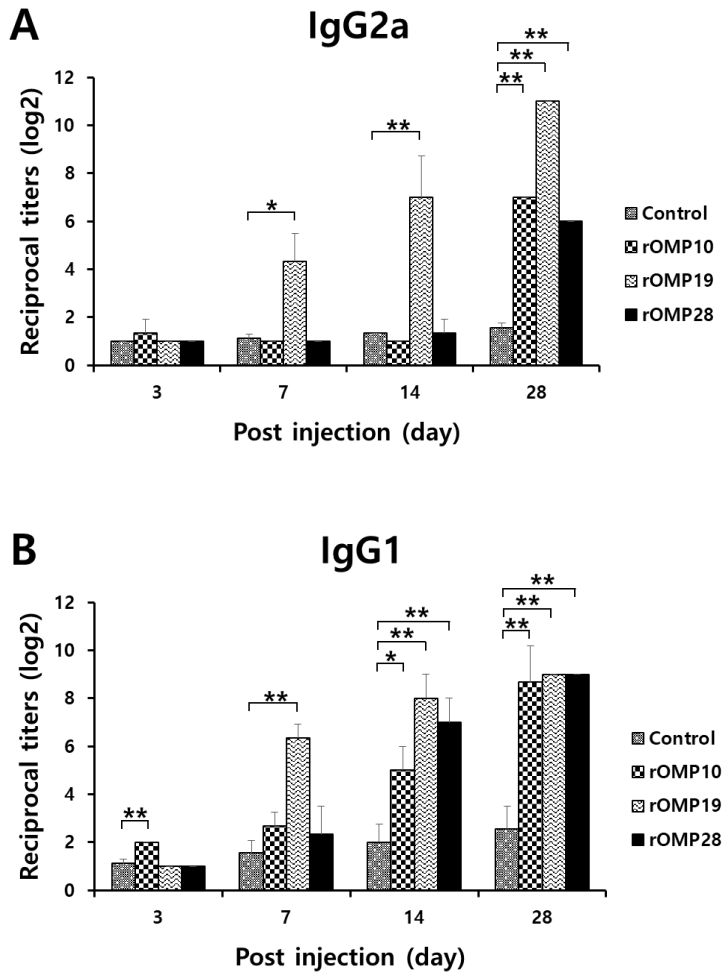


Figure 27. Humoral immune response induced IgG2a and IgG1. Production of IgG2a (A) was induced by immunization with rOMP19 after 7 days, while it was induced by rOMP10 and OMP28 after 28 days. Production of IgG1 (B) was induced immunization with OMP10 after 3 days, and OMP19 after 7 days. IgG1 production increased 14 days after stimulation with the 3 *B. abortus* antigens. (* $P < 0.05$, ** $P < 0.01$).

Chapter IV

Cytokines Production and Expression of Toll-like Receptors in Human Leukemic Monocyte Cells, THP-1, Stimulated with *Brucella abortus* Cellular Antigens

Abstract

A zoonotic pathogen, *Brucella* spp. is the causative agent of brucellosis, which results in abortion and loss in milk production in domestic animals, and undulant fever, osteoarticular pain and splenomegaly in humans. Due to the capability of the bacteria to modulate the host cell functions and survive in macrophages, early detection and eradication of the intracellular bacteria has received significant attention. Moreover, understanding the immunological alterations in *Brucella* infection is crucial to help develop control measures. Cytokines and toll-like receptors (TLRs) are some of the major compounds that play important roles in modulating the innate immunity and acquired immunity in host after infection. In

this study, therefore, human leukemic monocyte cells (THP-1 cells) were stimulated with five *B. abortus* cellular antigens: outer membrane protein 10 (OMP10), outer membrane protein 19 (OMP19), thiamine transporter substrate-binding protein (TbpA), arginase (RocF) and malate dehydrogenase (Mdh). Post stimulation, the cytokine productions and TLR expressions in the cells were evaluated at different time points (12 and 24 h), and analyzed using ELISA and real time RT-PCR, respectively. In the production of cytokines, it was observed that the production of TNF- α and IL-6 was highly induced in THP-1 cells stimulated with five *B. abortus* antigens. Also, TLR8 was induced in a time-dependent manner after stimulation with two *B. abortus* antigens, rOMP19 and rMdh, until 24 h. These results suggest that the two *B. abortus* antigens, rOMP19 and rMdh, might be involved in TLR8 signaling pathway in THP-1 cells in a time-dependent manner. These two *B. abortus* antigens are therefore potentially effective antigen candidates which would help to provide better understandings of the immune responses after *Brucella* infection.

Keywords: *Brucella abortus*, *B. abortus* antigens, Cytokines, TLRs, THP-1.

Introduction

Brucellosis is a serious zoonotic disease caused by the *Brucella* spp., a Gram-negative bacteria. It is indicated by undulant fever, endocarditis, arthritis and osteomyelitis in humans, and abortion and infertility in animals [Franco *et al.*, 2007]. Depending on the major preferred host and antigenic variation, the *Brucella* genus is classified into twelve species. *B. abortus* has received attention due to its significant clinical and economic importance. The *Brucella* spp. establish a chronic infection in the host cells by evasion of the host bactericidal phagocyte functions, and proliferate within the macrophages [Detilleux *et al.*, 1990a; Detilleux *et al.*, 1990b].

Although numerous researches have developed control measures including diagnostic methods against the bacterial infection through investigation of pathological pathways, several points still need to be addressed. Lipopolysaccharide (LPS) is regarded as the most important antigen during the immune response in brucellosis [Ko *et al.*, 2003]. Moreover, diagnosis of *B. abortus* in ruminant is mainly based on the serological confirmation with LPS-based antigens. The LPS-test based on this antigen could therefore give false positive results. Hence, many researchers have tried to develop diagnostic reagents without LPS [Al Dahouk *et al.*, 2006].

Recently, brucellosis has been actively investigated using the *Brucella* recombinant proteins and *Brucella* mutants for alternative diagnostic factors and vaccines [Boschiroli *et al.*, 1997; Wang *et al.*, 2011; Tan *et al.*, 2012]. Many research

groups have especially concentrated on studying to overcome the disadvantages of LPS-based diagnostics, and to develop more effective diagnostics and vaccines using various recombinant proteins. Among the recombinant proteins reported, the focus has been on the outer membrane protein28 (OMP28) has as an immunogen with high potency. Additionally, other *Brucella* cellular proteins such as OMP10, OMP19, OMP2b and SodC are reported to be effective antigens in inducing an immune response [Sung *et al.*, 2014; Simborio *et al.*, 2015; Im *et al.*, 2016b].

Cytokines have important roles in provoking the direction of immune responses, either cellular and/or humoral immunity. The cytokines, interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α), are produced in macrophages through the cellular immune response which induces the early immune responses [Zhan and Cheers, 1995]. Moreover, the productions of interferon gamma (IFN- γ) and interleukin 10 (IL-10) are known to be secreted from the CD4+ T cells in *Brucella* infection. These factors enhance the protection and/or alleviate the infection due to *Brucella*, via induction of the immune response [Stevens *et al.*, 1992; Ferenandes and Bladwin, 1995]. In humoral immunity, the levels of IgG, IgM, or IgA classes of antibodies are known to be important factors to help estimate the immune protection against *Brucella* infection. It can investigate the immune response which is changed by bacterial antigens invasion through the productions of the cytokines and antibodies.

Toll-like receptors (TLRs) are important factors in the immune response in cells, and are responsible for the signaling of infectious agents into cells. There are 10 TLR

families in humans, and 12 TLR families in mice. More specifically, TLR1-9 is common to both mouse and human. In mice, the function of TLR10 is limited, while in humans there is a role. In addition, TLR11-13 is present in mice while not present in humans [Kawai and Akira, 2010]. Of these, TLR4 is a known receptor related to cell apoptosis during infections, while TLR8 mediates the signals that inhibit cell apoptosis. Intracellular signaling pathways by *B. abortus* infection are mediated by mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF- κ B) [Gomes *et al.*, 2012]. This process is depending on the myeloid differentiation factor 88 (MyD88) and interleukin-1 receptor-associated kinases 4 (IRAK-4). Another study has further reported the importance of TLR9 in *B. abortus* infection [Gomes *et al.*, 2012].

This study, therefore, compared the changes in cytokine production and expression of genes involved in the TLR-related pathways in the intracellular immunity-inducing response of human leukemic monocyte cells, THP-1 cells, stimulated with *B. abortus* antigens, rMdh, rOMP10, rOMP19, rRocF and rTbpA. This research could be considered as basic data to further study the immunogenicity by intracellular invasion of *Brucella*.

Materials and Methods

Bacterial strains

The bacterial strains used in this study were *B. abortus* 544 (ATCC23448), a smooth virulent *B. abortus* biovar 1 strain and *E. coli* DH5 α (Invitrogen, USA). *B. abortus* was cultured in Brucella broth (BD Bioscience, USA), overnight at 37 °C in a gyratory shaker maintained at 220 rpm. *E. coli* DH5 α , were used to produce the necessary plasmid constructs; cultures were routinely grown at 37 °C in Luria-Bertani (LB) broth (Duchefa, Netherland) or agar supplemented with ampicillin (Sigma, USA). When solid medium and ampicillin were required, the above media were supplemented with 1.5 % (w/v) agar (Takara, Japan) and 100 μ g/ml of ampicillin (Sigma, USA).

Expression and purification of rOMP10, rOMP19, rTbpA, rRocF and rMdh proteins

The expression and purification of recombinant proteins was accomplished with a brief modification to a previously described [Sung *et al.*, 2014; Im *et al.*, 2016b]. Briefly, 20 ml of overnight cultured plasmid harboring *E. coli* was inoculated into 1 liter of LB broth containing ampicillin (25 μ g/ml) and cultured at 37 °C for 7 h. Protein expression was induced with isopropyl β -D-1-thiogalactopyranoside (IPTG, Amresco, USA) at a final concentration of 0.3 mM. Cells were then incubated at 37 °C for 2 h and harvested by centrifugation at 4,400 x g for 20 min. Cell pellets were resuspended in 40 ml column buffer (20 mM TrisHCl, 8 M Urea, 500 mM NaCl, 20 mM Imidazole, 1 mM β -mercaptoethanol, pH 8.0), sonicated at 10,000 Hz in an ice-water bath, and centrifuged at 4,400 x g for 20 min. Supernatants were loaded

onto a His SpinTrap (GE Healthcare, UK) column in accordance with the manufacturer's instructions. Purified proteins were stored at -20 °C until required.

SDS-PAGE and Western blot

SDS-PAGE and Western blotting were performed with the recombinant proteins. Briefly, the purified recombinant proteins were diluted with sample buffer and boiled for 10 min at 100 °C. After electrophoresis, samples were visualized by staining with Coomassie Brilliant Blue R-250 (Intron, Korea). Proteins resolved by SDS-PAGE were transferred to nitrocellulose membrane (Invitrogen, USA) for 25 min using an iBlot® transfer device (Invitrogen, USA). Membranes were blocked by incubating in 5 % skim milk (BD, USA) for 1 h at room temperature (RT) and washed 3 times with washing buffer (TBS) then, they were incubated with anti-histidine (1:2,000 dilution, AprilBio Co., Ltd, Korea) for 3 h at RT. The membranes were washed 3 times (10 min each) with the same washing buffer, after which they were incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (1:2,000 dilution, Bethyl, USA,) for 1 h at room temperature. After final washing, the proteins were visualized using on AP conjugate substrate kit (Bio-Rad, USA).

Human leukemic monocyte cell, THP-1, culture

The THP-1 human leukemic monocyte cell line was procured from the Korea Cell Line Bank (KCLB, Seoul, Korea), and cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS; Gibco, OK, USA) and antibiotic–

antimycotic solution (Sigma, MO, USA), at 37 °C in humidified air under 5 % CO₂. The THP-1 cells were differentiated into macrophages by stimulation with phorbol-12-myristate-13-acetate (PMA; Sigma, USA) (50 ng/ml) for 72 h, washed with FBS-free RPMI 1640 medium and incubated in 5 % FBS-RPMI 1640 medium without antibiotics for 24 h before the experiments.

Production of cytokines and expression of Toll-like receptors in THP-1 cells

ELISA was performed to evaluate 4 cytokines in the cell culture supernatant. The differentiated THP-1 macrophage cells (1×10^6 cells/ml) were stimulated with five *B. abortus* antigens (10 ug/well). The purified recombinant proteins were treated with polymyxin B (10 µg/ml) for 30 min before stimulation of cells. LPS (10 µg/ml, Sigma, USA) was included as positive control. It was slightly modified our previous studies [Sung *et al.*, 2014; Im *et al.*, 2016b]. The culture supernatants were collected at 12 and 24 h after stimulation. The amounts of TNF- α , IL-6, IL-1 β and IFN- γ were measured by ELISA according to the manufacturer's instructions (ThermoFisher, USA). Expression of TLRs in THP-1 macrophages was measured by quantitative real-time PCR. cDNA was synthesized from 1 µg of total RNA from each stimulated sample using a Quantitect® Reverse Transcription kit (Qiagen, Hilden, Germany), after which quantitative real-time RT-PCR was performed with 2 µl of cDNA using the Rotor-Gene SYBR Green PCR kit (Qiagen, Germany) and Rotor-Gene Q real-time PCR cycler (Qiagen, Germany) according to the manufacturer's protocols. The reaction mixture consisted of 10 µl of 2X Rotor-Gene SYBR Green PCR Master Mix,

2 µl of primers, 2 µl of template DNA or cDNA, and 6 µl of RNase-free water. The real-time PCR conditions for two TLR genes (TLR4 and TLR8), MyD88 and interferon regulatory factor 4 (IRF4) were used as follows: initial denaturation at 95 °C for 5 min followed by 45 cycles of 95 °C for 20s and annealing at 60 °C for 10s (Table 6). The gene expression level was normalized against expression of the beta-actin gene using the $2^{-\Delta\Delta C_t}$ formula, and relative expression levels of the target genes were compared with the control to determine changes in the fold expression of each gene.

Statistical analysis

Statistical significance (*P*-value) was calculated using the Student *t* test with the Statistical Package for Social Science (SPSS) software version 4.0 (MS, USA). Differences were considered to be significant if a value of $P < 0.05$. All experiments were repeated at least three times.

Results

Production levels of five recombinant proteins

Based on previous studies, five highly immunogenic cellular antigens of *B. abortus* were selected for evaluation from *B. abortus* antigens [Simborio *et al.*, 2015; Im *et al.*, 2016b; Reyes *et al.*, 2016; Im *et al.*, 2017]. Cloning of genes encoding Mdh,

OMP10, OMP19, RocF and TbpA was achieved as per a previous protocol, with a few modifications [Simborio *et al.*, 2015; Im *et al.*, 2016b; Im *et al.*, 2017]. *B. abortus* antigens were purified by a histidine column after expression of a trigger factor (TF) fusion protein using the TF cloning system. The SDS-PAGE profiles of the purified rOMP10, rOMP19, rTbpA, rRocF, and rMdh proteins were approximately 65.86 kDa, 71 kDa, 88.76 kDa, 85.19 kDa and 85.71 kDa in size, respectively (Figure 28A). Western blotting was also performed to confirm the specificity of the expressed proteins after protein purification, and the expected sizes of rMdh, rOMP10, rOMP19, rRocF, and rTbpA were evaluated (Figure 28B).

Production of cytokines in human leukemic monocytes cells, THP-1

Culture supernatants of THP-1 cells were assayed by ELISA for TNF- α , IL-1 β , IFN- γ and IL-6 production at 12 and 24 h after stimulation with rOMP10, rOMP19, rTbpA, rRocF and rMdh. The amount of TNF- α after stimulation with three *B. abortus* antigens (rOMP19, rTbpA and rMdh) was higher than the group stimulated with TF (Control) at 12 h. At 24 h post stimulation, four *B. abortus* antigen groups (rOMP19, rTbpA, rRocF and rMdh) showed significantly high production levels of TNF- α compared to the TF group ($P < 0.01$) (Figure 29A). Similarly, a significant amount of IL-6 was produced in the three *B. abortus* antigens exposure groups (rTbpA, rRocF and rMdh) at 12 h, whereas stimulation with all *B. abortus* antigens showed high production levels of IL-6 at 24 h, compared to the TF group ($P < 0.05$) (Figure 29D). However, production levels of the other cytokines (IL-1 β and IFN- γ)

were not significant when compared to the TF group and the other *B. abortus* antigen groups (Figure 29B, C). These results indicate that the five *B. abortus* antigens possess the Th1 and/or Th2 immune-stimulating properties in THP-1 cells, resulting in the production of TNF- α and IL-6. Furthermore, stimulation by two *B. abortus* antigens (rTbpA and rMdh) consistently induces the production of both cytokines (TNF- α and IL-6) in a time dependent manner, compared to the TF group.

Expression of TLRs in human leukemic monocyte cells, THP-1

THP-1 cells were harvested after stimulation with the five *B. abortus* antigens. RNA was isolated, followed by cDNA synthesis using the RNA template. Real-time PCR from TLR1 to TLR10 was performed to confirm expression levels of TLRs after stimulation with *B. abortus* antigens. Of the expression of TLRs, the expression of TLR3, TLR4, TLR7 and TLR8 were significantly increased after the stimulation of THP-1 cells by *B. abortus* antigens. Other TLRs did not show significant difference in the expression (data not shown). Expression of TLR3 was lower than that of TF at 12 h after stimulation with five *B. abortus* antigens. The expression levels of the four protein stimulating groups (rOMP10, rOMP19, rTbpA and rRocF) showed low expression levels except for the experimental group stimulated with rMdh until after 24 h (Figure 30A). At 12 h post stimulation, the expression of TLR4 was induced by two *B. abortus* antigens (rOMP10 and rRocF) (Figure 30B), whereas TLR8 expression was induced after stimulation with rOMP19 and rMdh (Figure 30D). In addition, TLR7 expression was observed to be higher in all experimental

groups at 24 h after stimulation with *B. abortus* antigens than the control group (Figure 30C). Expression of the MyD88 gene, which plays an important role in the TLRs signaling pathway, was also analyzed by real-time PCR. No significant difference were observed in the MyD88 gene expression between the experimental groups stimulated with *B. abortus* antigens and control group stimulated with TF protein (Figure 30E). The expression of IFN- γ regulatory factor (IRF) is one of the factors involved in the TLR signal pathway in cells. IRF4, a known inhibitor of IRF5, showed different expression levels in THP-1 cells stimulated with the five *B. abortus* antigens. rTbpA and rMdh induced higher expression levels of IRF4 than the TF group at 12 h after stimulation in THP-1 cells (Figure 30F). Furthermore, the rTbpA group consistently showed high expression levels after 24 h.

Discussion

The five *B. abortus* antigens used in this study have already been shown to have significant potent of immunogenicity in other studies [Simborio *et al.*, 2015; Im *et al.*, 2016b; Im *et al.*, 2017]. Each *B. abortus* antigens exhibits the following characteristics. rOMP10 and rOMP19 have been reported to have high efficacy in serologic evaluation and immune response studies [Tibor *et al.*, 1999; Simborio *et al.*, 2015]. rTbpA is known as a protein involved in metabolism in brucellosis [Im *et al.*, 2016b]. rRocF, arginase, is an enzyme that converts the substrate of iNOS

(arginine) into urea. rMdh is a protein involved in carbohydrate transport and metabolism and is known to be expressed by acidic stress [Lee *et al.*, 2015; Im *et al.*, 2017]. When THP-1 cells were stimulated with these *B. abortus* antigens, most of *B. abortus* antigens in the production levels of cytokines were higher than the control. However, when the results of cytokine production and TLR expression were integrated, rOMP19 and rMdh were found to be more promising candidates for analysis of immune response after stimulation.

It is important to observe changes in intracellular signal transduction and cytokine production during intracellular infection of *Brucella* spp. Numerous research groups have studied the immune response and new diagnostic candidates with LPS-free cellular components of *B. abortus* [Fu *et al.*, 2012; Lim *et al.*, 2012; Im *et al.*, 2017]. Various *B. abortus* cellular components, such as outer membrane proteins and cellular antigens, are effective for inducing of immune responses *in vitro* and *in vivo* [Lim *et al.*, 2012; Sung *et al.*, 2014; Simborio *et al.*, 2015; Im *et al.*, 2016b; Im *et al.*, 2017; Li *et al.*, 2018]. The induction of immune responses was assessed by analyzing the production levels of cytokines in the target cells after stimulation with bacterial pathogens. The intracellular immune response induces Th1 (cellular) or Th2 (humoral) immune response, depending on the role of the produced cytokines. The TNF- α and IFN- γ cytokines induce a Th1 immune response, whereas IL-6 elicits a Th2 immune response. Moreover, IL-6 has recently been shown to induce Th17 [De la Cruz-Mosso *et al.*, 2017; Zhao *et al.*, 2018]. In addition, comparative analysis of expression levels of IL-1 β , IL-12 and IL-18, which are typical for inducing

inflammatory responses in cells, has been widely used as an index for evaluating innate immunity. These results indicate that the cytokine production varied, depending on the cellular antigens of *B. abortus*. The production levels of TNF- α and IL-6 were higher than the other cytokines (IL-1 β and IFN- γ) in THP-1 cells stimulated with *B. abortus* antigens. This is similar to previous studies that reported cytokine production profiles in mouse macrophages, RAW 264.7 cells stimulated with *B. abortus* cellular antigens [Sung *et al.*, 2014; Im *et al.*, 2016b]. Moreover, our results might help to reveal the inherent mechanisms of *Brucella* infection, and the efficiency and immunogenicity of *B. abortus* cellular antigens.

Toll-like receptors are involved in intracellular signal transduction. Of the toll-like receptors studied, TLR2 and TLR4 in mice have an important effect on proinflammatory cytokine expression and cytotoxic T cell production from *Brucella* infection [Arias *et al.*, 2017]. In contrast, MyD88 present in the downstream of the TLR signaling pathway plays an important role in inducing immune responses in various cells such as lung, liver, and spleen. It is especially important in the spleen for controlling the immune response [Arias *et al.*, 2012]. TLR signal transduction is also important in studies using the human macrophage cells, THP-1 cells. TLR1 and TLR6 have been reported to affect the activity of nuclear factor kappa B (NF- κ B) after infection of pathogens [Shimizu *et al.*, 2008]. TLR8 plays an important role in inducing external antigen recognition and autoimmunity [Gantier *et al.*, 2010], and is also known to be expressed intracellularly. Specifically, it is activated by exogenous viral ssRNA, endogenous RNA, bacterial RNA and selfRNAs released

from dead or dying cell. Also, it is activated by bacterial RNA in human monocytes [Jorge *et al.*, 2012]. Furthermore, TLR8 recognize uridine-rich ssRNA certain chemical compounds with their distinct sites [Tanji *et al.*, 2015]. In this study, the difference in expression of TLR3, TLR4, TLR7 and TLR8 was dependent on the *B. abortus* antigens. However, in the overall TLR expression, the TLR8 expression is highly induced compared to TLR4 expression, which was assessed by evaluating the induction of immunity in THP-1 cells after stimulation with *B. abortus* antigens in this study. TLR8 is expressed in various tissues, with its highest expression in monocytes, and is upregulated after bacterial infection. Moreover, the expression of TLR3 and TLR7 in the endosome, TLR3 expression was lower than the control, whereas TLR7 expression was higher. TLR3 generally induces NF- κ B activity and cytokine expression through recognition of dsRNA and viruses. [Gomes *et al.*, 2012]. TLR7, some cases known to recognize the same ligand as TLR8, is known to recognize ssRNA and a nucleic acid-like structure of the virus [Kawai *et al.*, 2010]. Thus, TLR3, TLR4 and TLR7 are activated by *Brucella* infection and induce an immune response [Gomes *et al.*, 2012]. Interferon regulatory factor (IRF) 5 and 7 are known to interact with MyD88 and induce proinflammatory cytokines and type I interferons [Honda *et al.*, 2004; Takaoka *et al.*, 2005]. In contrast, IRF4 is known to interfere with the TLR signaling by competitively competing with the IRF5 to bind to MyD88 [Negishi *et al.*, 2005].

In conclusion, this study revealed that the five *B. abortus* antigens stimulate the immune activity in THP-1 cells. Of the *B. abortus* antigens, rOMP19 and rMdh

showed more effective activity than the others. These results indicate that two *B. abortus* antigens are possible candidates for further investigation in the pathogenesis of *Brucella* infection, and can be potential candidates for implementing measures to control the infection.

Table 6. Nucleotide sequence of primers used in this study

Gene	Primer sequence (5' - 3')	Annealing temperature (°C)	Product size (bp)	Reference
TLR1	F: TCATCCACGTTCCCTAAAGACCT R: GGGACAAATCCAAGTATTCCAA	60	198	[Im <i>et al.</i> , 2018b]
TLR2	F: CTCCAATCAGGCTTCTCTGTCT R: CACCTCTGTAGGTCAGTGTTC	60	159	[Im <i>et al.</i> , 2018b]
TLR3	F: CTCAGGTTTCAGCTGGAAAATCT R: TGAAAACACCCTGGAGAAAACCT	60	157	[Im <i>et al.</i> , 2018b]
TLR4	F: ATTGGCAGGAAGCAACATCTAT R: TCAATTCATTCTTCACCCACAG	60	162	[Im <i>et al.</i> , 2018b]
TLR5	F: GGACTTGACAACCTCCAAGTTC R: TCGGAGATCCAAGGTCTGTAAT	60	177	[Im <i>et al.</i> , 2018b]
TLR6	F: TCACCAGAGGTCCAACCTTACT R: ATTGTCAATGCTTTCAATGTCG	60	193	[Im <i>et al.</i> , 2018b]
TLR7	F: TTGAGAAGCCCTTTCAGAAGTC R: CACCTGACTATAGGCCACATGA	60	161	[Im <i>et al.</i> , 2018b]
TLR8	F: CAGATTTTGAGTTTGACCCACA R: ACAGGCAATGTCAGGAAGATTT	60	158	[Im <i>et al.</i> , 2018b]
TLR9	F: AGTTCTCTCTCCTGGCTGAATG R: GGACACCCTCTTTTGGTAATTG	60	171	[Im <i>et al.</i> , 2018b]
TLR10	F: CTGCTGTCTCCACTTTGATCTG R: TCAATTCATTCTTCACCCACAG	60	170	[Im <i>et al.</i> , 2018b]
MyD88	F: CGGATGGTGGTGGTTGTCTC R: CGCTTCTGATGGGCACCT	60	101	[Li <i>et al.</i> , 2013]
IRF4	F: GAAGCCTTGGCGTTCTCAGAC R: CGTATGTCCATGGGAGATCCG	60	117	[Li <i>et al.</i> , 2013]

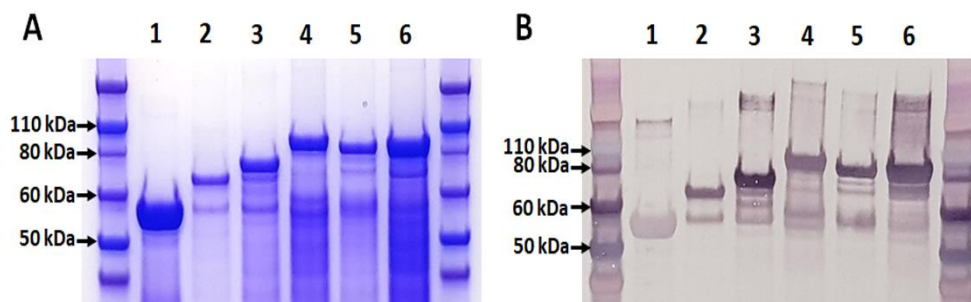


Figure 28. Analysis of *Brucella abortus* recombinant cellular proteins by SDS-PAGE (A) and Western blot (B). Lane 1: Control (TF; 52 kDa), Lane 2: rOMP10 (65.86 kDa), Lane 3: rOMP19 (71 kDa), Lane 4: rTbpA (88.76 kDa), Lane 5: rRocF (85.19 kDa), Lane 6: rMdh (85.71 kDa).

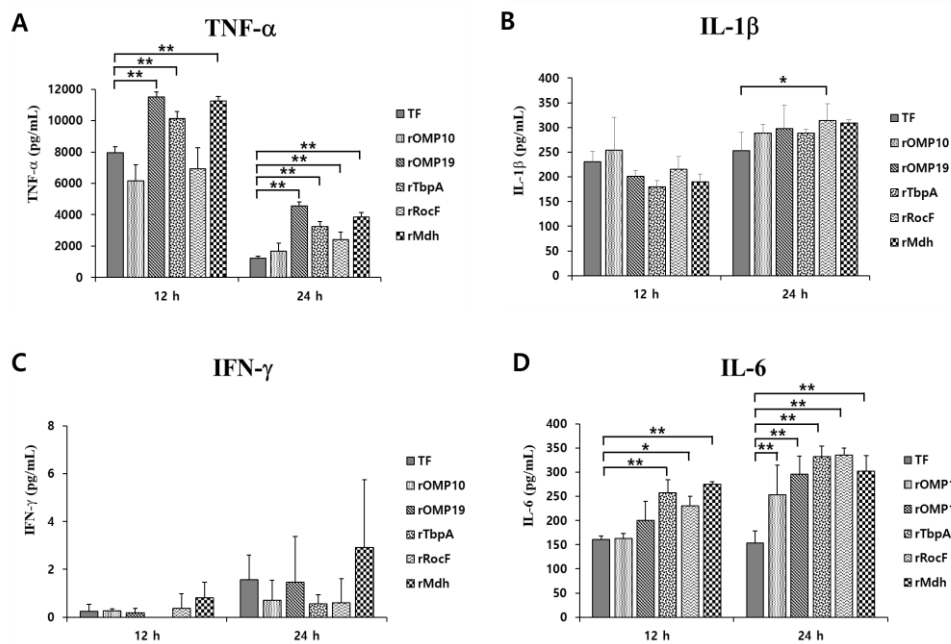


Figure 29. The production of cytokines in THP-1 cells (A) TNF- α , (B) IL-1 β , (C) IFN- γ and (D) IL-6, after stimulation with *B. abortus* antigens. TNF- α production was induced after stimulation with rOMP19, rTbpA and rMdh at 12 h and rOMP19, rTbpA, rRocF and rMdh at 24 h. IL-6 production was induced after stimulation with rTbpA, rRocF and rMdh at 12 h and rOMP10, rOMP19, rTbpA, rRocF and rMdh at 24 h. Production of IL-1 β and IFN- γ were not significantly induced at 12 and 24 h after stimulation with *B. abortus* antigens except the production of IL-1 β at 24 h after stimulation with rRocF. (* $P < 0.05$, ** $P < 0.01$).

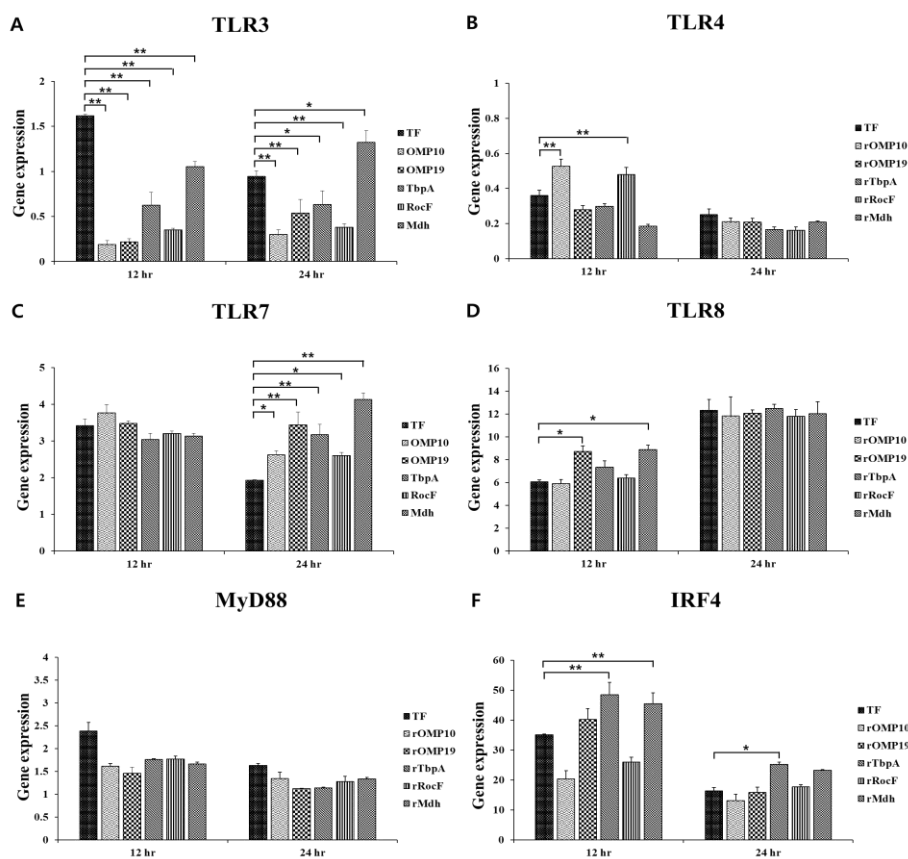


Figure 30. The expression of (A) TLR3, (B) TLR4, (C) TLR7, (D) TLR8, (E) MyD88 and (F) IRF4 in THP-1 cells after stimulation with *B. abortus* antigens. TLR3 expression was lower than that of TF at 12 and 24 h except for the expression level in the experimental group stimulated with rMdh. TLR4 expression increased after stimulation with rOMP10 and rRocF at 12 h. TLR7 expression was higher in all experimental groups than the control group at 24 h. TLR8 expression increased after stimulation with rOMP19 and rMdh at 12 h. The production of IRF4 increased at 12 h after stimulation with rTbpA and rMdh then decreased at 24 h. (* $P < 0.05$, ** $P < 0.01$).

General Conclusions

In an effort to solve the economic and social problems that arise from the onset of brucellosis, investigation of *Brucella* is steadily being conducted. Particularly, attempts to diagnose or prevent brucellosis through the early diagnosis of *Brucella* infection and the development of a vaccine have been continued by several researchers. Among these studies, efforts to develop diagnostic methods and vaccines using recombinant proteins of *Brucella* have become one of potent part of this area. In this study, *Brucella abortus* cellular antigens were used to stimulate various immune cells, analyze immune responses in each, and demonstrate excellent immunogenicity after stimulation with the antigens.

1. After stimulation of bPBMC with *B. abortus* antigens, the immune response was analyzed by gene expression of cytokines and apoptosis-related genes. As a result, it was observed that bPBMC can induce an appropriate immune response regardless of apoptosis. Although the gene expressions of iNOS, TNF- α and IL-1 β were not effective on bPBMC by the stimulation of *B. abortus* antigens, the gene expressions of IL-6, IL-12p40 and IFN- γ were significantly increased. These results indicate that the adaptive immune system is effectively activated in bPBMC by the stimulation of *B. abortus* antigens. In addition, it was confirmed that the expression of Bax and Bcl-2, which are apoptotic genes, is not affected except the expression of TLR4.

Therefore, *B. abortus* antigens are thought to induce an effective adaptive immune response in both humoral and cellular immunity without inducing an apoptosis.

2. In order to evaluate the immunogenicity of *B. abortus* antigens, rMdh, rRocF and rTsf, RAW 264.7 cells were stimulated the antigens. Also, the immune response was analyzed at BALB/c mice after immunization the antigens. As a result, *in vitro* test results showed that all three *B. abortus* antigens induce immune response in the cells. Especially, mouse splenocyte showed high expression of IFN- γ and IL-2 when stimulated with rMdh and rTsf. *In vivo* experiments indicated that IFN- γ - and IL-4-secreting cells were highly expressed, and that total IgG production was also elevated time-dependent after immunization ($P < 0.05$). These results showed that all of the three *B. abortus* antigens were able to confirm the potential as a high immunogenic antigen and induce the Th2 immune response well *in vivo*.

3. It was confirmed that TNF- α and IL-6 were highly produced by stimulation with *B. abortus* antigens, rOMP10, rOMP19 and rOMP28 in RAW 264.7 cells. Particularly, NO production level showed high at 24 h after the stimulation with rOMP19, and IFN- γ and IL-2 production were higher after the stimulation with rOMP28 compared to other *B. abortus* antigens. It is indicating that rOMP28 is expected to induce a Th1 immune response. *In vivo* experiments also confirmed that the three *B. abortus* antigens exhibited high IgG production. However, there was no difference in the production of IFN- γ - and IL-4-expressing T cells ($P < 0.05$).

Nevertheless, it shows the potential of the three *B. abortus* antigens as antigens by inducing immune responses that differ in the production of cytokines and IgG *in vitro* and *in vivo*.

4. Five of *B. abortus* antigens, which are already highly immunogenic, were selected and examined for immune responses in THP-1 cells after the stimulation. In the cytokine production, the productions of TNF- α and IL-6 were higher than that of IL-1 β and IFN- γ , which is similar to that in RAW 264.7 cells. In addition, real-time PCR confirmed the difference in the immune response by confirming changes in TLR expression. As a result, TLR3, -4, -7 and -8 showed different expression depending on *B. abortus* antigens. Especially, TLR8 was highly expressed at 12 h in the rOMP19 and rMdh stimulation groups. In addition, it was confirmed that TLR4 expression was low in *B. abortus* antigen groups (rTbpA and rMdh), which showed a high expression of IRF4, which interferes with the binding of MyD88 and IRF5 involved in TLR4 signaling ($P < 0.05$). Therefore, these two *B. abortus* antigens, rOMP19 and rMdh, showed the possibility for the high immunogenicity candidates in THP-1 cells, and further research on these two *B. abortus* antigens should be needed.

In conclusion, significant differences are showed with the expression of cytokines, IL-6, IL-12p40, and IFN- γ , in bPBMC, the productions of TNF- α and IL-6 in both RAW 264.7 and THP-1 cells after stimulation with *B. abortus* antigens. These results

indicated that a similar production of cytokines is induced after stimulation with the *B. abortus* antigens through analysis of the immune response of different host immune cells. In addition, different immune responses were observed depending on the *B. abortus* antigens in each host immune cells. In particular, among the *B. abortus* antigens used in this study, rMdh and rOMP19 showed high immunogenicity. Specifically, the immune responses of different immune cells, bPBMC, RAW 264.7 and THP-1 cells, were confirmed to be high in all experimental groups after stimulation with rMdh. Also, the rOMP19 stimulated groups showed high immunogenicity in RAW 264.7 and THP-1 cells. Therefore, these highly immunogenic antigens might be used for the development of brucellosis diagnosis and vaccine using the *Brucella* antigens. In addition, when the same *B. abortus* antigens are selected and the immune responses generated through different host immune cell stimuli were analyzed, it is considered that the antigen material exhibiting better immunogenicity can be selected. Therefore, the analysis of the host immune response using the *B. abortus* antigens could be an important step in the diagnosis, prevention and evaluation of the characteristics of brucellosis.

References

- Aggen, J.B., Armstrong, E.S., Goldblum, A.A., Dozzo, P., Linsell, M.S., Gliedt, M.J., Hildebrandt, D.J., Feeney, L.A., Kubo, A., Matias, R.D., Lopez, S., Gomez, M., Wlasichuk, K.B., Diokno, R., Miller, G.H., Moser, H.E., 2010. Synthesis and spectrum of the neoglycoside ACHN-490. *Antimicrob Agents Chemother* **54(11)**: 4636-4642.
- Ahmed, W., Zheng, K., Liu, Z.F., 2016. Establishment of chronic infection: *Brucella*'s stealth strategy. *Front Cell Infect Microbiol* **6**: 30.
- Al Dahouk, S., Nöckler, K., Scholz, H.C., Tomaso, H., Bogumil, R., Neubauer, H., 2006. Immunoproteomic characterization of *Brucella abortus* 1119-3 preparations used for the serodiagnosis of *Brucella* infection. *J Immunol Methods* **309(1-2)**: 34-47.
- Alva-Pérez, J., Arellano-Reynoso, B., Hernández-Castro, R., Suárez-Güemes, F., 2014. The *invA* gene of *Brucella melitensis* is involved in intracellular invasion and is required to establish infection in a mouse model. *Virulence* **5(4)**: 563-574.
- Aragón, V., Díaz, R., Moreno, E., Moriyón, I., 1996. Characterization of *Brucella abortus* and *Brucella melitensis* native haptens as outer membrane O-type polysaccharides independent from the smooth lipopolysaccharide. *J Bacteriol* **178(4)**: 1070-1079.
- Archambaud, C., Salcedo, S.P., Lelouard, H., Devillard, E., de Bovis, B., Van Rooijen, N., Gorvel, J.P., Malissen, B., 2010. Contrasting roles of macrophages and dendritic cells in controlling initial pulmonary *Brucella* infection. *Eur J Immunol* **40(12)**: 3458-3471.
- Arellano-Reynoso, B., Lapaque, N., Salcedo, S., Briones, G., Ciocchini, A.E., Ugalde, R., Moreno, E., Moriyón, I., Gorvel, J.P., 2005. Cyclic beta-1,2-glucan is a *Brucella*

- virulence factor required for intracellular survival. *Nat Immunol* **6(6)**: 618-625.
- Arias, M.A., Santiago, L., Costas-Ramon, S., Jaime-Sánchez, P., Freudenberg, M., Jiménez De Bagüés, M.P., Pardo, J.,** 2017. Toll-Like Receptors 2 and 4 cooperate in the control of the emerging pathogen *Brucella microti*. *Front Cell Infect Microbiol* **6**: 205.
- Baldi, P.C., Giambartolomei, G.H.,** 2013. Pathogenesis and pathobiology of zoonotic brucellosis in humans. *Rev Sci Tech* **32(1)**: 117-125.
- Baldi, P.C., Giambartolomei, G.H., Goldbaum, F.A., Abdón, L.F., Velikovsky, C.A., Kittelberger, R., Fossati, C.A.,** 1996. Humoral immune response against lipopolysaccharide and cytoplasmic proteins of *Brucella abortus* in cattle vaccinated with *B. abortus* S19 or experimentally infected with *Yersinia enterocolitica* serotype O:9. *Clin Diagn Lab Immunol* **3(4)**: 472-476.
- Baldwin, C.L., Goenka, R.,** 2006. Host immune responses to the intracellular bacteria *Brucella*: does the bacteria instruct the host to facilitate chronic infection?. *Crit Rev Immunol* **26(5)**: 407-442.
- Bao, Y., Tian, M., Li, P., Liu, J., Ding, C., Yu, S.,** 2017. Characterization of *Brucella abortus* mutant strain Δ 22915, a potential vaccine candidate. *Vet Res* **48(1)**: 17.
- Barlowe, C.,** 2002. COPII-dependent transport from the endoplasmic reticulum. *Curr Opin Cell Biol* **14(4)**: 417-422.
- Barquero-Calvo, E., Chaves-Olarte, E., Weiss, D.S., Guzman-Verri, C., Chacon-Diaz, C., Rucavado, A., Moriyon, I., Moreno, E.,** 2007. *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. *PLoS One* **2(7)**: e631.
- Barquero-Calvo, E., Conde-Alvarez, R., Chacón-Díaz, C., Quesada-Lobo, L.,**

- Martirosyan, A., Guzmán-Verri, C., Iriarte, M., Mancek-Keber, M., Jerala, R., Gorvel, J.P., Moriyón, I., Moreno, E., Chaves-Olarte, E., 2009.** The differential interaction of *Brucella* and ochrobactrum with innate immunity reveals traits related to the evolution of stealthy pathogens. *PLoS One* **4(6)**: e5893.
- Barquero-Calvo, E., Martirosyan, A., Ordoñez-Rueda, D., Arce-Gorvel, V., Alfaro-Alarcón, A., Lepidi, H., Malissen, B., Malissen, M., Gorvel, J.P., Moreno, E., 2013.** Neutrophils exert a suppressive effect on Th1 responses to intracellular pathogen *Brucella abortus*. *PLoS Pathog* **9(2)**: e1003167.
- Billard, E., Dornand, J., Gross, A., 2007.** *Brucella suis* prevents human dendritic cell maturation and antigen presentation through regulation of tumor necrosis factor alpha secretion. *Infect Immun* **75(10)**: 4980-4989.
- Boer, M.C., Prins, C., van Meijgaarden, K.E., van Dissel, J.T., Ottenhoff, T.H., Joosten, S.A., 2015.** *Mycobacterium bovis* BCG vaccination induces divergent proinflammatory or regulatory T cell responses in adults. *Clin Vaccine Immunol* **22(7)**: 778-788.
- Boschiroli, M.L., Cravero, S.L., Arese, A.I., Campos, E., Rossetti, O.L., 1997.** Protection against infection in mice vaccinated with a *Brucella abortus* mutant. *Infect Immun* **65(2)**: 798-800.
- Canning, P.C., Roth, J.A., Tabatabai, L.B., Deyoe, B.L., 1985.** Isolation of components of *Brucella abortus* responsible for inhibition of function in bovine neutrophils. *J Infect Dis* **152(5)**: 913-921.
- Celebi, G., Külah, C., Kiliç, S., Ustündağ, G., 2007.** Asymptomatic *Brucella* bacteraemia and isolation of *Brucella melitensis* biovar 3 from human breast milk. *Scand J Infect Dis* **39(3)**: 205-208.

- Celli, J., de Chastellier, C., Franchini, D.M., Pizarro-Cerda, J., Moreno, E., Gorvel, J.P.,** 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. *J Exp Med* **198(4)**: 545-556.
- Celli, J., Salcedo, S.P., Gorvel, J.P.,** 2005. *Brucella* coopts the small GTPase Sar1 for intracellular replication. *Proc Natl Acad Sci U S A* **102(5)**: 1673-1678.
- Cha, S.B., Rayamajhi, N., Kang, M.L., Lee, W.J., Shin, M.K., Yoo, H.S.,** 2010. Comparative study of gamma interferon production in mice immunized with outer membrane proteins and whole bacteria of *Brucella abortus*. *Jpn J Infect Dis* **63(1)**: 49-51.
- Chaudhuri, P., Prasad, R., Kumar, V., Gangaplara, A.,** 2010. Recombinant OMP28 antigen-based indirect ELISA for serodiagnosis of bovine brucellosis. *Mol Cell Probes* **24(3)**: 142-145.
- Cheers, C.,** 1984. Pathogenesis and cellular immunity in experimental murine brucellosis. *Dev Biol Stand* **56**: 237-246.
- Cloekaert, A., Jacques, I., Bosseray, N., Limet, J.N., Bowden, R., Dubray, G., Plommet, M.,** 1991. Protection conferred on mice by monoclonal antibodies directed against outer-membrane-protein antigens of *Brucella*. *J Med Microbiol* **34(3)**: 175-180.
- Comerci, D.J., Martínez-Lorenzo, M.J., Sieira, R., Gorvel, J.P., Ugalde, R.A.,** 2001. Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole. *Cell Microbiol* **3(3)**: 159-168.
- Conde-Álvarez, R., Arce-Gorvel, V., Gil-Ramírez, Y., Iriarte, M., Grilló, M.J., Gorvel, J.P., Moriyón, I.,** 2013. Lipopolysaccharide as a target for brucellosis vaccine design. *Microb Pathog* **58**: 29-34.
- Connolly, J.P., Comerci, D., Alefantis, T.G., Walz, A., Quan, M., Chafin, R., Grewal, P.,**

- Mujer, C.V., Ugalde, R.A., DelVecchio, V.G.,** 2006. Proteomic analysis of *Brucella abortus* cell envelope and identification of immunogenic candidate proteins for vaccine development. *Proteomics* **6(13)**: 3767-3780.
- Corbel, M.J.,** 1979. The relationship between the protective and cross-reacting antigens of *Brucella* spp., *Yersinia enterocolitica* O:9 and *Salmonella* serotypes of Kauffmann–White group N. *Contrib Microbiol Immunol* **5**: 50-63.
- Corbel, M.J.,** 1997. Brucellosis: an overview. *Emerg Infect Dis* **3(2)**: 213-221.
- Crawford, R.M., Van De Verg, L., Yuan, L., Hadfield, T.L., Warren, R.L., Drazek, E.S., Houn, H.H., Hammack, C., Sasala, K., Polsinelli, T., Thompson, J., Hoover, D.L.,** 1996. Deletion of *purE* attenuates *Brucella melitensis* infection in mice. *Infect Immun* **64(6)**: 2188-2192.
- de Figueiredo, P., Ficht, T.A., Rice-Ficht, A., Rossetti, C.A., Adams, L.G.,** 2015. Pathogenesis and immunobiology of brucellosis: review of *Brucella*-host interactions. *Am J Pathol* **185(6)**: 1505-1517.
- De la Cruz-Mosso, U., García-Iglesias, T., Bucala, R., Estrada-García, I., González-López, L., Cerpa-Cruz, S., Parra-Rojas, I., Gámez-Nava, J.I., Pérez-Guerrero, E.E., Muñoz-Valle, J.F.,** 2018. MIF promotes a differential Th1/Th2/Th17 inflammatory response in human primary cell cultures: Predominance of Th17 cytokine profile in PBMC from healthy subjects and increase of IL-6 and TNF- α in PBMC from active SLE patients, *Cell Immunol* **324**: 42-49.
- Delrue, R.M., Deschamps, C., Léonard, S., Nijskens, C., Danese, I., Schaus, J.M., Bonnot, S., Ferrooz, J., Tibor, A., De Bolle, X., Letesson, J.J.,** 2005. A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *Brucella melitensis*. *Cell Microbiol* **7(8)**: 1151-1161.

- Delrue, R.M., Martinez-Lorenzo, M., Lestrade, P., Danes, I., Bielarz, V., Mertens, P., De Bolle, X., Tibor, A., Gorvel, J.P., Letesson, J.J., 2001.** Identification of *Brucella* spp. genes involved in intracellular trafficking. *Cell Microbiol* **3(7)**: 487-497.
- Denoel, P.A., Crawford, R.M., Zygmunt, M.S., Tibor, A., Weynants, V.E., Godfroid, F., Hoover, D.L., Letesson, J.J., 1997.** Survival of a bacterioferritin deletion mutant of *Brucella melitensis* 16M in human monocyte-derived macrophages. *Infect Immun* **65(10)**: 4337-4340.
- Detilleux, P.G., Deyoe, B.L., Cheville, N.F., 1990a.** Entry and intracellular localization of *Brucella* spp. in Vero cells: fluorescence and electron microscopy. *Vet Pathol* **27(5)**: 317-328.
- Detilleux, P.G., Deyoe, B.L., Cheville, N.F., 1990b.** Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells *in vitro*. *Infect Immun* **58(7)**: 2320-2328.
- Diacovich, L., Gorvel, J.P., 2010.** Bacterial manipulation of innate immunity to promote infection. *Nat Rev Microbiol* **8(2)**: 117-128.
- Dornand, J., Gross, A., Lafont, V., Liautard, J., Oliaro, J., Liautard, J.P., 2002.** The innate immune response against *Brucella* in humans. *Vet Microbiol* **90(1-4)**: 383-394.
- Dorneles, E.M., Teixeira-Carvalho, A., Araújo, M.S., Sriranganathan, N., Lage, A.P., 2015.** Immune response triggered by *Brucella abortus* following infection or vaccination. *Vaccine* **33(31)**: 3659-3666.
- Ducken, D.R., Brown, W.C., Alperin, D.C., Brayton, K.A., Reif, K.E., Turse, J.E., Palmer, G.H., Noh, S.M., 2015.** Subdominant outer membrane antigens in *Anaplasma marginale*: conservation, antigenicity, and protective capacity using recombinant protein. *PLoS One* **10(6)**: e0129309.

- Durward, M., Radhakrishnan, G., Harms, J., Bareiss, C., Magnani, D., Splitter, G.A.,** 2012. Active evasion of CTL mediated killing and low quality responding CD8+ T cells contribute to persistence of brucellosis. *PLoS One* **7(4)**: e34925.
- Eckmann, L., Kangnoff, M.F.,** 2001. Cytokines in host defense against *Salmonella*. *Microbes Infect* **3(14-15)**: 1191-1200.
- Elenkov, I.J.,** 2004. Glucocorticoids and the Th1/Th2 balance. *Ann N Y Acad Sci* **1024**: 138-146.
- El-Sayed, A., Awad, W.,** 2018. Brucellosis: Evolution and expected comeback. *Int J Vet Sci Med* **6(Suppl)**: S31-S35.
- Endley, S., McMurray, D., Ficht, T.A.,** 2001. Interruption of the *cydB* locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. *J Bacteriol* **183(8)**: 2454-2462.
- Eskra, L., Mathison, A., Splitter, G.,** 2003. Microarray analysis of mRNA levels from RAW 264.7 macrophages infected with *Brucella abortus*. *Infect Immun* **71(3)**: 1125-1133.
- Ettema, T.J., Andersson, S.G.,** 2009. The alpha-proteobacteria: the Darwin finches of the bacterial world. *Biol Lett* **5(3)**: 429-432.
- Fahel, J.S., de Souza, M.B., Gomes, M.T., Corsetti, P.P., Carvalho, N.B., Marinho, F.A., de Almeida, L.A., Caliari, M.V., Machado, F.S., Oliveira, S.C.,** 2015. 5-Lipoxygenase negatively regulates Th1 response during *Brucella abortus* infection in mice. *Infect Immun* **83(3)**: 1210-1216.
- Fernandes, D.M., Baldwin, C.L.,** 1995. Interleukin-10 downregulates protective immunity to *Brucella abortus*. *Infect Immun* **63(3)**: 1130-1133.
- Fernandes, D.M., Benson, R., Baldwin, C.L.,** 1995. Lack of a role for natural killer cells in early control of *Brucella abortus* 2308 infections in mice. *Infect Immun* **63(10)**:

4029-4033.

- Ferrero, M.C., Fossati, C.A., Rumbo, M., Baldi, P.C., 2012.** *Brucella* invasion of human intestinal epithelial cells elicits a weak proinflammatory response but a significant CCL20 secretion. *FEMS Immunol Med Microbiol* **66(1)**: 45-57.
- Fontes, P., Alvarez-Martinez, M.T., Gross, A., Carnaud, C., Köhler, S., Liautard, J.P., 2005.** Absence of evidence for the participation of the macrophage cellular prion protein in infection with *Brucella suis*. *Infect Immun* **73(10)**: 6229-6236.
- Foster, G., Osterman, B.S., Godfroid, J., Jacques, I., Cloeckaert, A., 2007.** *Brucella ceti* sp. Nov. and *Brucella pinnipedialis* sp. Nov. for *Brucella* strains with cetaceans and seals as their preferred host. *Int J Syst Evol Microbiol* **57(Pt11)**: 2688–2693.
- Foulongne, V., Walravens, K., Bourg, G., Boschioli, M.L., Godfroid, J., Ramuz, M., O'Callaghan, D., 2001.** Aromatic compound-dependent *Brucella suis* is attenuated in both cultured cells and mouse models. *Infect Immun* **69(1)**: 547-550.
- Franco, M.P., Mulder, M., Gilman, R.H., Smith, H.L., 2007.** Human brucellosis. *Lancet Infect Dis* **7(12)**: 775-786.
- Freer, E., Moreno, E., Moriyón, I., Pizarro-Cerdá, J., Weintraub, A., Gorvel, J.P., 1996.** *Brucella-Salmonella* lipopolysaccharide chimeras are less permeable to hydrophobic probes and more sensitive to cationic peptides and EDTA than are their native *Brucella* spp. Counterparts. *J Bacteriol* **178(20)**: 5867-5876.
- Fu, S., Xu, J., Li, X., Xie, Y., Qiu, Y., Du, X., Yu, S., Bai, Y., Chen, Y., Wang, T., Wang, Z., Yu, Y., Peng, G., Huang, K., Huang, L., Wang, Y., Chen, Z., 2012.** Immunization of mice with recombinant protein CobB or AsnC confers protection against *Brucella abortus* infection. *PLoS One* **7(2)**: e29552.
- Fugier, E., Salcedo, S.P., de Chastellier, C., Pophillat, M., Muller, A., Arce-Gorvel, V.,**

- Fourquet, P., Gorvel, J.P.,** 2009. The glyceraldehyde-3-phosphate dehydrogenase and the small GTPase Rab 2 are crucial for *Brucella* replication. PLoS Pathog **5(6)**: e1000487.
- Gall, D., Nielsen, K., Nicola, A., Renteria, T.,** 2008. A proficiency testing method for detecting antibodies against *Brucella abortus* in quantitative and qualitative serological tests. Res Sci Tech **27(3)**: 819–828.
- Gantier, M.P., Irving, A.T., Kaparakis-Liaskos, M., Xu, D., Evans, V.A., Cameron, P.U., Bourne, J.A., Ferrero, R.L., John, M., Behlke, M.A., Williams, B.R.,** 2010. Genetic modulation of TLR8 response following bacterial phagocytosis. Hum Mutat **31(9)**: 1069-1079.
- Gao, N., Jennings, P., Guo, Y., Yuan, D.,** 2011. Regulatory role of natural killer (NK) cells on antibody responses to *Brucella abortus*. Innate Immun **17(2)**: 152-163.
- Ghasemi, A., Jeddi-Tehrani, M., Mautner, J., Salari, M.H., Zarnani, A.H.,** 2014. Immunization of mice with a novel recombinant molecular chaperon confers protection against *Brucella melitensis* infection. Vaccine **32(49)**: 6659-6666.
- Ghasemi, A., Zarnani, A.H., Ghoojani, A., Rezania, S., Salari, M.H., Jeddi-Tehrani, M.,** 2014. Identification of a new immunogenic candidate conferring protection against *Brucella melitensis* infection in mice. Mol Immunol **62(1)**: 142-149.
- Giambartolomei, G.H., Zwerdling, A., Cassataro, J., Bruno, L., Fossati, C.A., Philipp, M.T.,** 2004. Lipoproteins, not lipopolysaccharide, are the key mediators of the proinflammatory response elicited by heat-killed *Brucella abortus*. J Immunol **173(7)**: 4635-4642.
- Goel, D., Bhatnagar, R.,** 2012. Intradermal immunization with outer membrane protein 25 protects BALB/c mice from virulent *B. abortus* 544. Mol Immunol **51(2)**: 159-168.

- Gogoi, M., Datey, A., Wilson, K.T., Chakravortty, D.,** 2016. Dural role of arginine metabolism in establishing pathogenesis. *Curr Opin Microbiol* **29**: 43-48.
- Goldbaum, F.A., Leoni, J., Wallach, J.C., Fossati, C.A.,** 1993. Characterization of an 18-kilodalton *Brucella* cytoplasmic protein which appears to be a serological marker of active infection of both human and bovine brucellosis. *J Clin Microbiol* **31(8)**: 2141-2145.
- Golding, B., Scott, D.E., Scharf, O., Huang, L.Y., Zaitseva, M., Lapham, C., Eller, N., Golding, H.,** 2001. Immunity and protection against *Brucella abortus*. *Microbes Infect* **3(1)**: 43-48.
- Gomes, M.T., Campos, P.C., de Almeida, L.A., Oliveira, F.S., Costa, M.M., Marim, F.M., Pereira, G.S., Oliveira, S.C.,** 2012. The role of innate immune signals in immunity to *Brucella abortus*. *Front Cell Infect Microbiol* **2**: 130.
- Gomez, G., Adams, L.G., Rice-Ficht, A., Ficht, T.A.,** 2013. Host-*Brucella* interactions and the *Brucella* genome as tools for subunit antigen discovery and immunization against brucellosis. *Front Cell Infect Microbiol* **3**: 17.
- González-Smith, A., Vemulapalli, R., Andrews, E., Oñate, A.,** 2006. Evaluation of *Brucella abortus* DNA vaccine by expression of Cu-Zn superoxide dismutase antigen fused to IL-2. *Immunobiology* **211(1-2)**: 65-74.
- Grillo, M.J., Blasco, J.M., Gorvel, J.P., Moriyon, I., Moreno, E.,** What have we learned from brucellosis in the mouse model?. *Vet Res* **43(1)**: 29.
- Gu, W., Wang, X., Qiu, H., Cui, B., Zhao, S., Zheng, H., Xiao, Y., Liang, J., Duan, R., Jing, H.,** 2003. Comparison of cytokine immune responses to *Brucella abortus* and *Yersinia enterocolitica* serotype O:9 infections in BALB/c mice. *Infect Immun* **81(12)**: 4392-4398.

- Guzmán-Verri, C., Chaves-Olarte, E., von Eichel-Streiber, C., López-Goñi, I., Thelestam, M., Arvidson, S., Gorvel, J.P., Moreno, E., 2001.** GTPases of the Rho subfamily are required for *Brucella abortus* internalization in nonprofessional phagocytes: direct activation of Cdc42. *J Biol Chem* **276(48)**: 44435-44443.
- Hamer, I., Goffin, E., De Bolle, X., Letesson, J.J., Jadot, M., 2014.** Replication of *Brucella abortus* and *Brucella melitensis* in fibroblast does not require Atg5-dependent macroautophagy. *BMC Microbiol* **14**: 223.
- Hamid, N., Jain, S.K., 2008.** Characterization of an outer membrane protein of *Salmonella enterica* serovar Typhimurium that confers protection against typhoid. *Clin Vaccine Immunol* **15(9)**: 1461-1471.
- Han, X., Tong, Y., Tian, M., Sun, X., Wang, S., Ding, C., Yu, S., 2014.** Characterization of the immunogenicity and pathogenicity of malate dehydrogenase in *Brucella abortus*. *World J Microbiol Biotechnol* **30(7)**: 2063-2070.
- Hicks, C.A., Willi, B., Riond, B., Novacco, M., Meli, M.L., Stokes, C.R., Helps, C.R., Hofmann-Lehmann, R., Tasker, S., 2015.** Protective immunity against infection with *Mycoplasma haemofelis*. *Clin Vaccine Immunol* **22(1)**: 108-118.
- Hielpos, M.S., Fernández, A.G., Falivene, J., Alonso Paiva, I.M., Muñoz González, F., Ferrero, M.C., Campos, P.C., Vieira, A.T., Oliveira, S.C., Baldi, P.C., 2018.** IL-1R and inflammasomes mediate early pulmonary protective mechanisms in respiratory *Brucella abortus* infection. *Front Cell Infect Microbiol* **8**: 391.
- Honda, K., Yanai, H., Mizutani, T., Negishi, H., Shimada, N., Suzuki, N., Ohba, Y., Takaoka, A., Yeh, W.C., Taniguchi, T., 2004.** Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proc Natl Acad Sci U S A* **101(43)**: 15416-15421.

- Hop, H.T., Simborio, H.L., Reyes, A.W., Arayan, L.T., Min, W., Lee, H.J., Kim, D.H., Chang, H.H., Kim, S.,** 2015. Immunogenicity and protective effect of recombinant *Brucella abortus* Ndk (rNdk) against a virulent strain *B. abortus* 544 infection in BALB/c mice. *FEMS Microbiol Lett* **362(4)**: doi: 10.1093/femsle/fnv003.
- Huang, L.Y., Aliberti, J., Leifer, C.A., Segal, D.M., Sher, A., Golenbock, D.T., Golding, B.,** 2003. Heat-killed *Brucella abortus* induces TNF and IL-12p40 by distinct MyD88-dependent pathways: TNF, unlike IL-12p40 secretion, is Toll-like receptors dependent. *J Immunol* **171(3)**: 1441-1446.
- Iannino, F., Ugalde, J.E., de Iannino, N.I.,** 2012. *Brucella abortus* *efp* gene is required for an efficient internalization in HeLa cells. *Microb Pathog* **52(1)**: 31-40.
- Im, Y.B., Jung, M., Shin, M.K., Kim, S., Yoo, H.S.,** 2016a. Expression of cytokine and apoptosis-related genes in bovine peripheral blood mononuclear cells stimulated with *Brucella abortus* recombinant proteins. *Vet Res* **47**: 30.
- Im, Y.B., Park, W.B., Jung, M., Kim, S., Yoo, H.S.,** 2016b. Evaluation of Th1/Th2-related immune response against recombinant proteins of *Brucella abortus* infection in mice. *J Microbiol Biotechnol* **26(6)**: 1132-1139.
- Im, Y.B., Park W.B., Jung, M., Kim, S., Yoo, H.S.,** 2018a. Comparative analysis of immune responses to outer membrane antigens OMP10, OMP19, and OMP28 of *Brucella abortus*. *Jpn J Infect Dis* **71(3)**: 197-204.
- Im, Y.B., Shim, S., Soh, S.H., Kim, S., Yoo, H.S.,** 2018b. Cytokines production and toll-like receptors expression in human leukemic monocyte cells, THP-1, stimulated with *Brucella abortus* cellular antigens. *Microb Pathog* **122**: 7-12.
- Im, Y.B., Shim, S., Park, W.B., Kim, S., Yoo, H.S.,** 2017. Th2-related immune responses by the *Brucella abortus* cellular antigens, malate dehydrogenase, elongation factor,

- and arginase. *Microb Pathog* **110**: 7-13.
- Iwakabe, K., Shimada, M., Ohta, A., Yahata, T., Ohmi, Y., Habu, S., Nishimura, T.,** 1998. The restraint stress drives a shift in Th1/Th2 balance toward Th2-dominant immunity in mice. *Immunol Lett* **62(1)2**: 39-43.
- Jiang, X., Leonard, B., Benson, R., Baldwin, C.L.,** 1993. Macrophage control of *Brucella abortus*: role of reactive oxygen intermediates and nitric oxide. *Cell Immunol* **151(2)**: 309-319.
- Jiménez de Bagüés, M.P., Marín, C.M., Blasco, J.M., Moriyón, I., Gamazo, C.,** 1992. An ELISA with *Brucella* lipopolysaccharide antigen for the diagnosis of *B. melitensis* infection in sheep and for the evaluation of serological responses following subcutaneous or conjunctival *B. melitensis* strain Rev 1 vaccination. *Vet Microbiol* **30(2-3)**: 233-241.
- Jorge, L.C., Bennett, W., Chaitali, B., Juan, C.S.,** 2012. TLR8: the forgotten relative revindicated. *Cell Mol Immunol* **9(6)**: 434-438.
- Jung, M., Shim, S., Im, Y.B., Park, W.B., Yoo, H.S.,** 2018. Global gene-expression profiles of intracellular survival of the BruAb2_1031 gene mutated *Brucella abortus* in professional phagocytes, RAW 264.7 cells. *BMC Microbiol* **18(1)**: 82.
- Kapsenberg, M.L.,** Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol* **3(12)**: 984-993.
- Kawai, T., Akira, S.,** 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11(5)**: 373-384.
- Kianmehr, Z., Soleimanjahi, H., Ardestani, S.K., Fotouhi, F., Abdoli, A.,** 2015. Influence of *Brucella abortus* lipopolysaccharide as an adjuvant on the immunogenicity of HPV-16 L1VLP vaccine in mice. *Med Microbiol Immunol* **204(2)**: 205-213.

- Kim, J.Y., Sung, S.R., Lee, K.C., Lee, H.K., Kang, S.I., Lee, J.J., Jung, S.C., Park, Y.H., Her, M.,** 2014. Immunoproteomics of *Brucella abortus* RB51 as candidate antigens in serological diagnosis of brucellosis. *Vet Immunol Immunopathol* **160(3-4)**: 218-224.
- Kim, S., Watarai, M., Suzuki, H., Makino, S., Kodama, T., Shirahata, T.,** 2004. Lipid raft microdomains mediate class A scavenger receptor-dependent infection of *Brucella abortus*. *Microb Pathog* **37(1)**: 11-19.
- Kim, W.K., Moon, J.Y., Cho, J.S., Hur, J.,** 2017. Protective efficacy by various doses of a new brucellosis vaccine candidate based on *Salmonella* strains expressing *Brucella abortus* BSCP31, Omp3b and superoxide dismutase against brucellosis in murine model. *Pathog Dis* **75(7)**: ftx094.
- Kittelberger, R., Bundesen, P.G., Cloeckaert, A., Greiser-Wilke, I., Letesson, J.J.,** 1998. Serological cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* O:9. IV. Evaluation of the M- and C-epitope antibody response for the specific detection of *B. abortus* infections. *Vet Microbiol* **60(1)**: 45-57.
- Ko, J., Gendron-Fitzpatrick, A., Ficht, T.A., Splitter, G.A.,** 2002. Virulence criteria for *Brucella abortus* strains as determined by interferon regulatory factor 1-deficient mice. *Infect Immun* **70(12)**: 7004-7012.
- Ko, J., Splitter, G.A.,** 2003. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. *Clin Microbiol Rev* **16(1)**: 65-78.
- Ko, K.Y., Kim, J.W., Her, M., Kang, S.I., Jung, S.C., Cho, D.H., Kim, J.Y.,** 2012. Immunogenic proteins of *Brucella abortus* to minimize cross reactions in brucellosis diagnosis. *Vet Microbiol* **156(3-4)**: 374-380.

- Konnai, S., Usui, T., Ohashi, K., Onuma, M.,** 2003. The rapid quantitative analysis of bovine cytokine genes by real-time RT-PCR. *Vet Microbiol* **94(4)**: 283-294.
- Kovach, M.E., Elzer, P.H., Robertson, G.T., Chirhart-Gilleland, R.L., Christensen, M.A., Peterson, K.M., Roop, R.M. 2nd.,** 1997. Cloning and nucleotide sequence analysis of a *Brucella abortus* gene encoding an 18 kDa immunoreactive protein. *Microb Pathog.* **22(4)**: 241-246.
- Lapaque, N., Muller, A., Alexopoulou, L., Howard, J.C., Gorvel, J.P.,** 2009. *Brucella abortus* induces Irgm3 and Irga6 expression via type-I IFN by a MyD88-dependent pathway, without the requirement of TLR2, TLR4, TLR5 and TLR9. *Microb Pathog* **47(6)**: 299-304.
- Lee, J.J., Simborio, H.L., Reyes, A.W.B., Kim, D.G., Hop, H.T., Min, W.G., Her, M., Jung, S.C., Yoo, H.S., Kim, S.,** 2014. Proteomic analyses of the time course responses of mice infected with *Brucella abortus* 544 reveal immunogenic antigens. *FEMS Microbiol Lett* **357(2)**: 164-174.
- Lee, J.J., Simborio, H.L., Reyes, A.W.B., Kim, D.G., Hop, H.T., Min, W.G., Her, M., Jung, S.C., Yoo, H.S., Kim, S.,** 2015. Immunoproteomic identification of immunodominant antigenic independent of the time of infection in *Brucella abortus* 2308-challenged cattle. *Vet Res* **46**: 17.
- LeVier, K., Phillips, R.W., Grippe, V.K., Roop 2nd, R.M., Walker, G.C.,** 2000. Similar requirements of a plant symbiont and a mammalian pathogen for prolonged intracellular survival. *Science* **287(5462)**: 2492-2493.
- Li, B., Baylink, D.J., Deb, C., Zannetti, C., Rajaallah, F., Xing, W., Walter, M.H., Lau, K.H., Qin, X.,** 2013. 1,25-Dihydroxyvitamin D3 suppresses TLR8 expression and TLR8-mediated inflammatory responses in monocytes *in vitro* and experimental

- autoimmune encephalomyelitis *in vivo*. PLoS One **8(3)**: e58808.
- Li, J.Y., Liu, Y., Gao, X.X., Gao, X., Cai, H.**, 2014. TLR2 and TLR4 signaling pathways are required for recombinant *Brucella abortus* BCSP31-induced cytokine production, functional upregulation of mouse macrophages, and the Th1 immune response *in vivo* and *in vitro*. Cell Mol Immunol **11(5)**: 478–494.
- Li, M., Li, C., Song, S., Kang, H., Yang, D., Li, G.**, 2016. Development and antigenic characterization of three recombinant proteins with potential for Glässer's disease prevention. Vaccine **34(19)**: 2251-2258.
- Li, P., Tian, M., Bao, Y., Hu, H., Liu, J., Yin, Y., Ding, C., Wang, S., Yu, S.**, 2017. *Brucella* rough mutant induce macrophage death via activating IRE1 α Pathway of endoplasmic reticulum stress by enhanced T4SS secretion. Front Cell Infect Microbiol **7**: 422.
- Li, Z., Fu, Q., Wang, Z., Li, T., Zhang, H., Guo, F., Wang, Y., Zhang, J., Chen, C.**, 2015. TceSR two-component regulatory system of *Brucella melitensis* 16M is involved in invasion, intracellular survival and regulated cytotoxicity for macrophages. Lett Appl Microbiol **60(6)**: 565-571.
- Li, Z., Wang, S., Zang, H., Xi, L., Zang, J., Zang, X., Zhou, Q., Yi, J., Li, M., Zhang, W., Zhang, J.**, 2018. Development and evaluation of in murine model, of an improved live-vaccine candidate against brucellosis from to *Brucella melitensis* vjbR deletion mutant. Microb Pathog **124**: 250-257.
- Li, Z., Zhang, H., Zhang, J., Xi, L., Yang, G., Wang, S., Zhou, Q., Zhang, X., Zhang, J.**, 2018. *Brucella abortus* phosphoglyceromutase and dihydrodipicolinate reductase induce Th1 and Th2-related immune responses. World J Microbiol Biotechnol **34(2)**: 22.

- Lim, J.J., Kim, D.H., Lee, J.J., Kim, D.G., Min, W., Lee, H.J., Rhee, M.H., Chang, H.H., Kim, S.,** 2012a. Evaluation of recombinant 28 kDa outer membrane protein of *Brucella abortus* for the clinical diagnosis of bovine brucellosis in Korea. J Vet Med Sci **74(6)**: 687-691.
- Lim, J.J., Kim, D.H., Lee, J.J., Kim, D.G., Min, W., Lee, H.J., Rhee, M.H., Kim, S.,** 2012b. Protective effects of recombinant *Brucella abortus* Omp28 against infection with a virulent strain of *Brucella abortus* 544 in mice. J Vet Sci **13(3)**: 287-292.
- Lindler, L.E., Hadfield, T.L., Tall, B.D., Snellings, N.J., Rubin, F.A., Van De Verg, L.L., Hoover, D., Warren, R.L.,** 1996. Cloning of a *Brucella melitensis* group 3 antigen gene encoding Omp28, a protein recognized by the humoral immune response during human brucellosis. Infect Immun **64(7)**: 2490-2499.
- Liu, Z., Yuan, F., Yang, Y., Yin, L., Liu, Y., Wang, Y., Zheng, K., Cao, J.,** 2016. Partial protective immunity against toxoplasmosis in mice elicited by recombinant *Toxoplasma gondii* malate dehydrogenase. Vaccine **34(7)**: 989-994.
- Lowry, J.E., Goodridge, L., Vernati, G., Fluegel, A.M., Edwards, W.H., Andrews, G.P.,** 2010. Identification of *Brucella abortus* genes in elk (*Cervus elaphus*) using *in vivo*-induced antigen technology (IVIAT) reveals novel markers of infection. Vet Microbiol **14(3-4)**: 367-372.
- Luo, Y., O'Hagan, D., Zhou, H., Singh, M., Ulmer, J., Reisfeld, R.A., James Primus, F., Xiang, R.,** 2003. Plasmid DNA encoding human carcinoembryonic antigen (CEA) adsorbed onto cationic microparticles induces protective immunity against colon cancer in CEA-transgenic mice. Vaccine **21(17-18)**: 1938-1947.
- Manterola, L., Guzmán-Verri, C., Chaves-Olarte, E., Barquero-Calvo, E., de Miguel, M.J., Moriyón, I., Grilló, M.J., López-Goñi, I., Moreno, E.,** 2007. BvrR/BvrS-

- controlled outer membrane proteins Omp3a and Omp3b are not essential for *Brucella abortus* virulence. Infect Immun **75(10)**: 4867-4874.
- Martínez de Tejada, G., Pizarro-Cerdá, J., Moreno, E., Moriyón, I.**, 1995. The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. Infect Immun **63(8)**: 3054-3061.
- Martirosyan, A., Gorvel, J.P.**, 2013. *Brucella* evasion of adaptive immunity. Future Microbiol **8(2)**: 147-154.
- Martirosyan, A., Moreno, E., Gorvel, J.P.**, 2011. An evolutionary strategy for a stealthy intracellular *Brucella* pathogen. Immunol Rev **240(1)**: 211-234.
- McGiven, J.A.**, 2013. New development in the immunodiagnosis of brucellosis in livestock and wildlife. Res Sci Tech **32(1)**: 163-176.
- McGiven, J.A., Stack, J.A., Perrett, L.L., Tucker, J.D., Brew, S.D., Stubberfield, E., MacMillan, A.P.**, 2006. Harmonisation of European tests for serological diagnosis of *Brucella* infection in bovines. Rev Sci Tech **25(3)**: 1039-1053.
- Meltzer, E., Sidi, Y., Smolen, G., Banai, M., Bardenstein, S., Schwartz, E.**, 2010. Sexually transmitted brucellosis in humans. Clin Infect Dis **51(2)**: e12-15.
- Mol, J.P., Costa, E.A., Carvalho, A.F., Sun, Y.H., Tsolis, R.M., Paixão, T.A., Santos, R.L.**, 2014. Early transcriptional responses of bovine chorioallantoic membrane explants to wild type, $\Delta virB2$ or $\Delta btpB$ *Brucella abortus* infection. PLoS One **9(9)**: e108606.
- Montero, D., Orellana, P., Gutiérrez, D., Araya, D., Salazar, J.C., Prado, V., Oñate, A., Del Canto, F., Vidal, R.**, 2014. Immunoproteomic analysis to identify Shiga toxin-producing *Escherichia coli* outer membrane proteins expressed during human infection. Infect Immun **82(11)**: 4767-4777.
- Moreno, E., Jones, L.M., Berman, D.T.**, 1984. Immunochemical characterization of rough

- Brucella* lipopolysaccharides. Infect Immun **43(3)**: 779-782
- Negishi, H., Ohba, Y., Yanai, H., Takaoka, A., Honma, K., Yui, K., Matsuyama, T., Taniguchi, T., Honda, K., 2005.** Negative regulation of Toll-like-receptor signaling by IRF-4. Proc Natl Acad Sci U S A **102(44)**: 15989-15994.
- Nielsen, K., Smith, P., Widdison, J., Gall, D., Kelly, L., Kelly, W., Nicoletti, P., 2004.** Serological relationship between cattle exposed to *Brucella abortus*, *Yersinia enterocolitica* O:9 and *Escherichia coli* O157:H7. Vet Microbiol **100(1-2)**: 25-30.
- Nielsen, K., Smith, P., Yu, W.L., Halbert, G., 2007.** *Salmonella enterica* serotype Urbana interference with brucellosis serology. J Immunoassay Immunochem **28(3)**: 289-296.
- Nielsen, K., Yu, W.L., 2010.** Serological diagnosis of brucellosis. Prilozi **31(1)**: 65-89.
- Nieves, W., Heang, J., Asakrah, S., Höner zu Bentrup, K., Roy, C.J., Morici, L.A., 2010.** Immunospecific responses to bacterial elongation factor Tu during *Burkholderia* infection and immunization. PLoS One **5(12)**: e14361.
- Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K., Akira, S., 2000.** Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. J Immunol **164(7)**: 3476-3479.
- O'Callaghan, D., Cazeville, C., Allardet-Servent, A., Boschirol, M.L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y., Ramuz, M., 1999.** A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol Microbiol **33(6)**: 1210-1220.
- Okagawa, T., Konnai, S., Mekata, H., Githaka, N., Suzuki, S., Kariuki, E., Gakuya, F.,**

- Kanduma, E., Shirai, T., Ikebuchi, R., Ikenaka, Y., Ishizuka, M., Murata, S., Ohashi, K.,** 2012. Transcriptional profiling of inflammatory cytokine genes in African buffaloes (*Syncerus caffer*) infected with *Theileria parva*. *Vet Immunol Immunopathol* **148(3-4)**: 373-379.
- Oñate, A.A., Vemulapalli, R., Andrews, E., Schurig, G.G., Boyle, S., Folch, H.,** 1999. Vaccination with live *Escherichia coli* expressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. *Infect Immun* **67(2)**: 986-988.
- Paixao, T.A., Roux, C.M., Den Hartigh, A.B., Sankaran-Walters, S., Dandekar, S., Santos, R.L., Tsolis, R.M.,** 2009. Establishment of systemic *Brucella melitensis* infection through the digestive tract requires urease, the type IV secretion system, and lipopolysaccharide. *Infect Immun* **77(10)**: 4197-4208.
- Pappas, G.,** 2010. The changing *Brucella* ecology: novel reservoirs, new threats. *Int J Antimicrob Agents* **36(Suppl 1)**: S8-11.
- Páramo, L., Lomonte, B., Pizarro-Cerdá, J., Bengoechea, J.A., Gorvel, J.P., Moreno, E.,** 1998. Bactericidal activity of Lys49 and Asp49 myotoxic phospholipases A2 from Bothrops asper snake venom--synthetic Lys49 myotoxin II-(115-129)-peptide identifies its bactericidal region. *Eur J Biochem* **253(2)**: 452-461.
- Parent, M.A., Goenka, R., Murphy, E., Levier, K., Carreiro, N., Golding, B., Ferguson, G., Roop, R.M. 2nd., Walker, G.C., Baldwin, C.L.,** 2007. *Brucella abortus bacA* mutant induces greater pro-inflammatory cytokines than the wild-type parent strain. *Microbes Infect* **9(1)**: 55-62.
- Park, W.B., Im, Y.B., Shim, S., Yoo, H.S.,** 2018. Analysis of protein expression in *Brucella abortus* mutants with different growth rates by two-dimensional gel electrophoresis

- and LC-MS/MS peptide analysis. *J Vet Sci* **19(2)**: 216-231.
- Pei, J., Ding, X., Fan, Y., Rice-Ficht, A., Ficht, T.A.**, 2012. Toll-like receptors are critical for clearance of *Brucella* and play different roles in development of adaptive immunity following aerosol challenge in mice. *Front Cell Infect Microbiol* **2**: 115.
- Pizarro-Cerdá, J., Desjardins, M., Moreno, E., Akira, S., Gorvel, J.P.**, 1999. Modulation of endocytosis in nuclear factor IL-6(-/-) macrophages is responsible for a high susceptibility to intracellular bacterial infection. *J Immunol* **162(6)**: 3519-3526.
- Pizarro-Cerdá, J., Méresse, S., Parton, R.G., van der Goot, G., Sola-Landa, A., Lopez-Goñi, I., Moreno, E., Gorvel, J.P.**, 1998. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. *Infect Immun* **66(12)**: 5711-5724.
- Plommet, M., Plommet, A.M.**, 1988. Virulence of *Brucella*: bacterial growth and decline in mice. *Ann Vet Res* **19(1)**: 65-67.
- Poester, F.P., Samartino, L.E., Santos, R.L.**, 2013. Pathogenesis and pathobiology of brucellosis in livestock. *Rev Sci Tech* **32(1)**: 105-115.
- Porte, F., Liautard, J.P., Köhler, S.**, 1999. Early acidification of phagosomes containing *Brucella suis* is essential for intracellular survival in murine macrophages. *Infect Immun* **67(8)**: 4041-4047.
- Quebatte, M., Dehio, M., Tropel, D., Basler, A., Toller, I., Raddatz, G., Engel, P., Huser, S., Schein, H., Lindroos, H.L., Andersson, S.G., Dehio, C.**, 2010. The BatR/BatS two-component regulatory system controls the adaptive response of *Bartonella henselae* during human endothelial cell infection. *J Bacteriol* **192(13)**: 3352-3367.
- Reyes, A.W., Simborio, H.L., Hop, H.T., Arayan, L.T., Kim, S.**, 2016. Molecular cloning, purification and immunogenicity of recombinant *Brucella abortus* 544 malate

- dehydrogenase protein. J Vet Sci **17(1)**: 119-122.
- Reyes, A.W., Hop, H.T., Arayan, L.T., Huy, T.X., Min, W., Lee, H.J., Chang, H.H., Kim, S.,** 2017. Nocodazole treatment interrupted *Brucella abortus* invasion in RAW 264.7 cells, and successfully attenuated splenic proliferation with enhanced inflammatory response in mice. Microb Pathog **103**: 87-93.
- Ruiz-Mesa, J.D., Sánchez-Gonzalez, J., Reguera, J.M., Martín, L., Lopez-Palmero, S., Colmenero, J.D.,** 2005. Rose bengal test: diagnostic yield and use for the rapid diagnosis of human brucellosis in emergency departments in endemic area. Clin Microbiol Infect **11(3)**: 211-215.
- Salcedo, S.P., Marchesini, M.I., Lelouard, H., Fugier, E., Jolly, G., Balor, S., Muller, A., Lapaque, N., Demaria, O., Alexopoulou, L., Commerci, D.J., Ugalde, R.A., Pierre, P., Gorvel, J.P.,** 2008. *Brucella* control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. PLoS Pathog **4(2)**: e21.
- Scholz, H.C., Hubalek, Z., Sedláček, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H., Cloeckert, A., Maquart, M., Zygmunt, M.S., Whatmore, A.M., Falsen, E., Bahn, P., Göllner, C., Pfeffer, M., Huber, B., Busse, H.J., Nöckler, K.,** 2008. *Brucella microti* spp. nov., isolated from the common vole *Microtus arvalis*. Int J Syst Evol Microbiol **58(Pt 2)**: 375–382.
- Scholz, H.C., Nöckler, K., Göllner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al Dahouk, S., Kämpfer, P., Cloeckert, A., Maquart, M., Zygmunt, M.S., Whatmore, A.M., Pfeffer, M., Huber, B., Busse, H.J., De, B.K.,** 2010. *Brucella inopinata* spp. nov., isolated from a breast implant infection. Int J Syst Evol Microbiol **60(Pt4)**: 801–808.
- Scholz, H.C., Revilla-Fernández, S., Al Dahouk, S., Hammerl, J.A., Zygmunt, M.S.,**

- Cloeckaert, A., Koylass, M., Whatmore, A.M., Blom, J., Vergnaud, G., Witte, A., Aistleitner, K., Hofer, E., 2016.** *Brucella vulpis* spp. nov., isolated from mandibular lymph nodes of red foxes (*Vulpes vulpes*). Int J Syst Evol Microbiol **66(5)**: 2090–2098.
- Scian, R., Barrionuevo, P., Fossati, C.A., Giambartolomei, G.H., Delpino, M.V., 2012.** *Brucella abortus* invasion of osteoblasts inhibits bone formation. Infect Immun **80(7)**: 2333-2345.
- Seleem, M.N., Boyle, S.M., Sriranganathan, N., 2010.** Brucellosis: a re-emerging zoonosis. Vet Microbiol **140(3-4)**: 392-398.
- Sharma, J., Mishra, B.B., Li, Q., Teale, J.M., 2011.** TLR4-dependent activation of inflammatory cytokines response in macrophages by *Francisella* elongation factor Tu. Cell Immunol **269(2)**: 69-73.
- Shim, S., Im, Y.B., Jung, M., Park, W.B., Yoo, H.S., 2018.** Genes related to intracellular survival of *Brucella abortus* in THP-1 macrophage cells. J Microbiol Biotechnol **28(10)**: 1736-1748.
- Simborio, H.L., Lee, J.J., Bernardo Reyes, A.W., Hop, H.T., Arayan, L.T., Min, W., Lee, H.J., Yoo, H.S., Kim, S., 2015.** Evaluation of the combined use of the recombinant *Brucella abortus* Omp10, Omp19 and Omp28 proteins for the clinical diagnosis of bovine brucellosis. Microb Pathog **83-84**: 41-46.
- Shimizu, T., Kida, Y., Kuwano, K., 2008.** *Ureaplasma parvum* lipoproteins, including MB antigen, activate NF- κ B through TLR1, TLR2 and TLR6. Microbiology **154(Pt 5)**: 1318-1325.
- Snyder, G.A., Deredge, D., Waldhuber, A., Fresquez, T., Wilkins, D.Z., Smith, P.T., Durr, S., Cirl, C., Jiang, J., Jennings, W., Luchetti, T., Snyder, N., Sundberg, E.J.,**

- Wintrode, P., Miethke, T., Xiao, T.S.,** 2014. Crystal structures of the Toll/Interleukin-1 receptor (TIR) domains from the *Brucella* protein TcpB and host adaptor TIRAP reveal mechanisms of molecular mimicry. *J Biol Chem* **289(2)**: 669-679.
- Soh, S.H., Shim, S., Im, Y.B., Park, H.T., Cho, C.S., Yoo, H.S.,** 2018. Induction of Th2-related immune responses and production of systemic IgA in mice intranasally immunized with *Brucella abortus* malate dehydrogenase loaded chitosan nanoparticles. *Vaccine* **37(12)**: 1554-1564.
- Sola-Landa, A., Pizarro-Cerdá, J., Grilló, M.J., Moreno, E., Moriyón, I., Blasco, J.M., Gorvel, J.P., López-Goñi, I.,** 1998. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. *Mol Microbiol* **29(1)**: 125-138.
- Sowa, B.A., Kelly, K.A., Ficht, T.A., Frey, M., Adams, L.G.,** 1991. SDS-soluble and peptidoglycan-bound proteins in the outer membrane-peptidoglycan complex of *Brucella abortus*. *Vet Microbiol* **27(3-4)**: 351-369.
- Stephens, D.J., Lin-Marq, N., Pagano, A., Pepperkok, R., Paccaud, J.P.,** 2000. COPI-coated ER-to-Golgi transport complexes segregate from COPII in close proximity to ER exit sites. *J Cell Sci* **113(Pt12)**: 2177-2185.
- Stevens, M.G., Pugh, G.W. Jr., Tabatabai, L.B.,** 1992. Effects of gamma interferon and indomethacin in preventing *Brucella abortus* infections in mice. *Infect Immun* **60(10)**: 4407-4409.
- Sung, K.Y., Jung, M., Shin, M.K., Park, H.E., Lee, J.J., Kim, S., Yoo, H.S.,** 2014. Induction of immune responses by two recombinant proteins of *Brucella abortus*, outer membrane proteins 2b porin and Cu/Zn superoxide dismutase, in mouse model.

- J Microbiol Biotechnol **24(6)**: 854-861.
- Sung, K.Y., Yoo, H.S.**, 2014. Host immune responses during *Brucella* infection: a brief review. J Prev Vet Med **38**: 26-34.
- Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T.W., Taniguchi, T.**, 2005. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature **434(7030)**: 243-249.
- Tan, W., Wang, X.R., Nie, Y., Wang, C., Cheng, L.Q., Wang, X.C., Zhang, R., Yan, G.M.**, 2012. Recombinant VirB5 protein as a potential serological marker for the diagnosis of bovine brucellosis. Mol Cell Probes **26(3)**: 127–131.
- Tanji, H., Ohto, U., Shibata, T., Taoka, M., Yamauchi, Y., Isobe, T., Miyake, K., Shimizu, T.**, 2015. Toll-like receptor 8 senses degradation products of single-stranded RNA. Nat Struct Mol Biol **22(2)**: 109-115.
- Teixeira-Gomes, A.P., Cloeckaert, A., Zygmunt, M.S.**, 2000. Characterization of heat, oxidative, and acid stress responses in *Brucella melitensis*. Infect Immun **68(5)**: 2954-2961.
- Tian, M., Qu, J., Han, X., Ding, C., Wang, S., Peng, D.**, 2014. Mechanism of Asp24 upregulation in *Brucella abortus* rough mutant with a disrupted O-antigen export system and effect of Asp24 in bacterial intracellular survival. Infect Immun **82(7)**: 2840-2850.
- Tian, M., Qu, J., Han, X., Zhang, Ding, M., Ding, C., Ding, J.**, 2013. Microarray-based identification of differentially expressed genes in intracellular *Brucella abortus* within RAW 264.7 cells. PLoS One **8(8)**: e67014.
- Tibor, A., Decelle, B., Letesson, J.J.**, 1999. Outer membrane proteins Omp10, Omp16, and

- Omp19 of *Brucella* spp. are lipoproteins. Infect Immun **67(9)**: 4960-4962.
- Tibor, A., Saman, E., de Wergifosse, P., Cloeckaert, A., Limet, J.N., Letesson, J.J., 1996.**
Molecular characterization, occurrence, and immunogenicity in infected sheep and cattle of two minor outer membrane proteins of *Brucella abortus*. Infect Immun **64(1)**: 100-107.
- Tiwari, S., Kumar, A., Thavaselvam, D., Mangalgi, S., Rathod, V., Prakash, A., Barua, A., Arora, S., Sathyaseelan, K., 2013.** Development and comparative evaluation of a plate enzyme-linked immunosorbent assay based on recombinant outer membrane antigens Omp28 and Omp31 for diagnosis of human brucellosis. Clin Vaccine Immunol **20(8)**: 1217-1222.
- Truong, Q.L., Cho, Y., Park, S., Kim, K., Hahn, T.W., 2016.** *Brucella abortus* $\Delta cydC\Delta cydD$ and $\Delta cydC\Delta purD$ double-mutants are highly attenuated and confer long-term protective immunity against virulent *Brucella abortus*. Vaccine **34(2)**: 237-244.
- Vishnu, U.S., Sankarasubramanian, J., Gunasekaran, P., Rajendhran, J., 2015.**
Novel vaccine candidates against *Brucella melitensis* identified through reverse vaccinology approach. OMICS **19(11)**: 722-729.
- Vitry, M.A., Hanot Mambres, D., Deghelt, M., Hack, K., Machelart, A., Lhomme, F., Vanderwinden, J.M., Vermeersch, M., De Trez, C., Pérez-Morga, D., Letesson, J.J., Muraille, E., 2014.** *Brucella melitensis* invades murine erythrocytes during infection. Infect Immun **82(9)**: 3927-3938.
- Wang, Y., Bai, Y., Qu, Q., Xu, J., Chen, Y., Zhong, Z., Qiu, Y., Wang, T., Du, X., Wang, Z., Yu, S., Fu, S., Yuan, J., Zhen, Q., Yu, Y., Chen, Z., Huang, L., 2011.** The 16M Δ vjbR as an ideal live attenuated vaccine candidate for differentiation between

- Brucella* vaccination and infection. Vet Microbiol **151(3-4)**: 354-362.
- Watarai, M., Makino, S., Fujii, Y., Okamoto, K., Shirahata, T., 2002.** Modulation of *Brucella*-induced macropinocytosis by lipid rafts mediates intracellular replication. Cell Microbiol **4(6)**: 341-355.
- Weiss, D.S., Takeda, K., Akira, S., Zychlinsky, A., Moreno, E., 2005.** MyD88, but not toll-like receptors 4 and 2, is required for efficient clearance of *Brucella abortus*. Infect Immun **73(8)**: 5137-5143.
- Whatmore, A.M., Davison, N., Cloeckaert, A., Al Dahouk, S., Zygmunt, M.S., Brew, S.D., Perrett, L.L., Koylass, M.S., Vergnaud, G., Quance, C., Scholz, H.C., Dick, E.J. Jr., Hubbard, G., Schlabritz-Loutsevitch, N.E., 2014.** *Brucella papionis* spp. nov., isolated from baboons (*Papio* spp.). Int J Syst Evol Microbiol **64(Pt12)**: 4120–4128.
- Xu, D., Song, J., Li, G., Cai, W., Zong, S., Li, Z., Liu, W., Hu, S., Bu, Z., 2018.** A novel small RNA Bmsr1 enhances virulence in *Brucella melitensis* M28. Vet Microbiol **223**: 1-8.
- Xu, X., Zhang, K., Huang, Y., Ding, L., Chen, G., Zhang, H., Tong, D., 2012.** Bovine herpes virus type 1 induces apoptosis through Fas-dependent and mitochondria-controlled manner in Madin–Darby bovine kidney cells. Virol J **9**: 202.
- Young, E.J., 1995.** An overview of human brucellosis. Clin Infect Dis **21(2)**: 283-289.
- Zaitseva, M., Golding, H., Manischewitz, J., Webb, D., Golding, B., 1996.** *Brucella abortus* as a potential vaccine candidate: induction of interleukin-12 secretion and enhanced B7.1 and B7.2 and intercellular adhesion molecule 1 surface expression in elutriated human monocytes stimulated by heat-inactivated *B. abortus*. Infect Immun **64(8)**: 3109-3117.

- Zhan, Y., Cheers, C., 1995a.** Differential induction of macrophage-derived cytokines by live and dead intracellular bacteria *in vitro*. *Infect Immun* **63(2)**: 720-723.
- Zhan, Y., Kelso, A., Cheers, C., 1993.** Cytokine production in the murine response to *Brucella* infection or immunization with antigenic extracts. *Immunology* **80(3)**: 458-464.
- Zhan, Y., Kelso, A., Cheers, C., 1995.** Differential activation of *Brucella*-reactive CD4⁺ T cells by *Brucella* infection or immunization with antigenic extracts. *Infect Immun* **63(3)**: 969-975.
- Zhang, J., Yin, S., Yi, D., Zhang, H., Li, Z., Guo, F., Chen, C., Fang, W., Wang, J., 2017.** The *Brucella melitensis* M5-90ΔmanB live vaccine candidate is safer than M5-90 and confers protection against wild-type challenge in BALB/c mice. *Microb Pathog* **112**: 148-155.
- Zhao, M., Tan, Y., Peng, Q., Huang, C., Guo, Y., Liang, G., Zhu, B., Huang, Y., Liu, A., Wang, Z., Li, M., Gao, X., Wu, R., Wu, H., Long, H., Lu, Q., 2018.** IL-6/STAT3 pathway induced deficiency of RFX1 contributes to Th17-dependent autoimmune diseases via epigenetic regulation. *Nat Commun* **9(1)**: 583.

국문초록

*Brucella abortus*의 항원 자극에 의한 면역반응

임 영 빈

(지도교수: 유 한 상, D.V.M., Ph.D.)

서울대학교 대학원

수의학과 수의병인생물학 및 예방수의학 전공

브루셀라증은 브루셀라 속균감염에 의해 지속적으로 발생되고 있는 주요한 인수공통전염병 중 하나이다. 특히, *Brucella abortus*는 소에서 유·사산, 불임등의 번식장애를 사람에서는 파상열, 심내막염, 관절염, 및 골수염을 유발한다. 이러한 *B. abortus*는 유·사산된 태아, 태반, 양수 및 감염동물의 유선을 통해서 배출된다. *B. abortus*의 감염은 유·사산물과의 직·간접적인 접촉 또는 멸균되지 않은 유제품의 섭취등에 의해서 이루어질 수 있다.

다양한 경로를 통하여 숙주세포에 침투한 *B. abortus*는 숙주세포의 면역기전을 회피하기 위한 자가보호시스템을 구축하고 있으며, *Brucella*-containing vacuole (BCV), Type IV secretion system (T4SS), Cyclic β -1,2-glucan (c β G), *Brucella* lipopolysaccharide (Br-LPS)등의 여러가지 요소들을 이용하여 세포내에서의 생존/복제가 가능하다. 또한, 숙주세포에서 면역반응으로 발현되는 사이토카인의 발현을 조절하기 위하여 다양한 대식세포들의 신호전달체계를 방해한다. 이러한 세포내 침입과 생존/복제가 가능한 브루셀라의 예방 및 근절을 위하여 많은 연구가 진행되었다. 특히, 숙주세포 침입기전 분석 및 면역학적 특성 분석에 관한 연구에 많이 집중되었다.

현재 브루셀라의 초기 감염을 진단하기 위한 노력은 지속되고 있으며 *B. abortus* 감염시 강력한 면역반응을 유발시키는 *B. abortus* 항원들이 진단항원으로써 주목을 받고 있다. 본 연구에서는 이러한 *B. abortus* 세포항원들을 서로 다른 동물유래의 면역세포에서 면역유도능력을 사이토카인과 면역관련 유전자의 발현차이로 비교하여, *B. abortus* 감염증 예방을 위해 발병기전, 최적의 진단법 및 백신항원후보물질 발굴을 위한 기초 자료를 제공하고자 하였다.

첫번째로, 소의 말초혈액단핵세포(PBMC)의 면역반응 양상을 알아보고자 IL-1 β , IL-4, IL-6, IL-12p40, IFN- γ , TNF- α 및 iNOS의 발현을 확인하였다. 또한, 세가지 세포사멸 관련 유전자인 Bax, Bcl-2 및 TLR4의 발현을 *B.*

abortus 항원(rMdh, rOMP28, rRocF, rTsf and r0628) 자극에 의한 면역세포의 반응을 통하여 분석하였다. 각 *B. abortus* 항원은 자극 시간 및 투여량에 따라 사이토카인 발현양상은 상이하게 나타났다. 즉, IL-1 β , IL-4, TNF- α 및 iNOS 유전자 발현은 관찰되지 않았지만, IL-6, IL-12p40 및 IFN- γ 의 발현은 모든 *B. abortus* 항원 자극으로 유도되었다($P < 0.05$). 세포사멸 관련 유전자의 발현은 TLR4 외에 변화가 없었다($P < 0.05$).

두번째로, 마우스 유래의 면역세포인 RAW 264.7 세포 및 마우스에 *B. abortus* 항원(rMdh, rOMP10, rOMP19, rOMP28, rRocF and rTsf)을 자극하여 면역반응의 차이를 분석하였다. 사이토카인 발현은 *B. abortus* 항원으로 RAW 264.7 세포와 비장세포를 자극시킨 후 조사하였다. 면역 반응은 *B. abortus* 항원(rMdh, rOMP10, rOMP19, rOMP28, rRocF and rTsf)으로 마우스를 면역시킨 후 ELISA 및 ELISpot을 통하여 비교분석하였다. 자극된 RAW 264.7 세포에서는 TNF- α 와 IL-6 발현이 증가하였고($P < 0.05$), 비장세포에서는 Th1 관련 사이토카인 IFN- γ 와 IL-2가 유도되었다($P < 0.01$). 또한, 마우스 면역에 따른 생체내 면역반응을 IgG 분비 세포의 발현 차이를 분석함으로써 *B. abortus* 항원에 따라 유도되는 면역반응을 비교한 결과 면역 후 28일째 IgG의 발현이 현저하게 증가하는 것을 관찰할 수 있었다($P < 0.05$).

마지막으로, 사람유래 면역세포인 THP-1 세포를 *B. abortus* 항원(rMdh,

rOMP10, rOMP19, rRocF and rTbpA)으로 자극하여 발생하는 면역반응의 차이를 확인하였다. 특히, 사이토카인과 Toll-like receptors (TLRs) 같은 선천성 면역을 조절하고, 감염 후 숙주가 면역을 획득하는데 중요한 역할을 하는 주요 조절인자들의 발현차이에 초점을 맞추어 진행하였다. *B. abortus* 항원으로 THP-1 세포를 자극 시킨 후, ELISA 및 real-time RT-PCR을 사용하여 사이토카인 생성 및 TLR 발현량을 두시점(12 및 24시간)에서 정량, 비교분석하였다. 사이토카인 생성에 있어서, *B. abortus* 항원으로 자극된 THP-1 세포에서 TNF- α 및 IL-6의 발현량이 높게 유도되었다($P < 0.05$). 또한, TLR8은 rOMP19 및 rMdh로 자극 후 12시간에 대조군에 비해 유의한 발현증가를 나타냈다. 이러한 결과는 두 개의 *B. abortus* 항원인 rOMP19와 rMdh가 THP-1 세포에서의 TLR8 발현유도에 관한 추가 연구의 필요성을 시사한다.

B. abortus 항원(rMdh, rOMP10, rOMP19, rOMP28, rRocF, rTbpA, rTsf and r0628)을 이용한 서로 다른 동물유래 면역세포들의 면역반응 연구결과를 종합하면, 본 연구에 사용된 *B. abortus* 항원들 중 고면역원성을 나타내는 수종의 *B. abortus* 세포 항원이 확인되었다. 이들 *B. abortus* 항원 중 rMdh와 rOMP19는 높은 면역원성을 나타내었다. 특히, rMdh는 본 연구에서 사용한 모든 동물유래 면역세포에 대해 고면역원성을 보였다. 본 연구를 통하여 *B. abortus* 항원의 세포내 면역반응 조절인자로서의 활성뿐 아니

라 고면역원성 항원 후보물질로서의 가능성을 확인할 수 있었으며, 이러한 연구결과는 향후 *B. abortus* 감염증 예방을 위한 진단법 개발 및 백신 후보물질 발굴을 위한 기초자료로 유용할 것으로 사료된다.

핵심어: 브루셀라증, 브루셀라, *B. abortus* 항원, 면역반응, Th1, Th2, 사이토카인, 면역세포.

학번: 2010-31124